

How to give good dogs bad names

The British nuclear industry has been given a year to clean up its leaky separation plant, but it will take longer to repair the damage the leaks have done to the reputation of nuclear power.

THERE is nothing quite like the nuclear industry for fossilizing the technology of the past. A nuclear installation, once commissioned, becomes radioactive and thus inaccessible by people, with the result that radical improvements of performance are virtually impossible. This simple truth lies at the root of last week's fierce criticism, by Britain's Nuclear Installations Inspectorate, of the reprocessing plant at Windscale (now called Sellafield) in Cumbria, on the north-east seaboard of England (see p. 603). The plant was designed in a hurry nearly 40 years ago, soon after Britain decided to use plutonium for nuclear bombs. The plant reprocessing the bulk of spent nuclear fuel from British nuclear reactors was built nearly 30 years ago, and afterwards extended in bits and pieces. Throughout the life of the plant, heroic efforts have been made to keep it working safely and successfully, but they have not prevented a long string of embarrassing mishaps that would have seemed comic were they not also potentially tragic. Now the nuclear inspectors have rightly said that this must stop, and the Health and Safety Executive has used its legal powers to tell British Nuclear Fuels (BNFL), the publicly owned company that operates the plant, that it has a year in which to put its house in order. "High time, too", will be the general reaction.

So it is, but for a variety of reasons not all as simple as the evident and understandable exasperation of the nuclear inspectors' report suggests. It goes without saying that the mishaps of technical incompetence belie the now-forgotten boast of British nuclear engineers in the 1950s that their techniques would set standards of excellence which, when followed by the rest of British industry, would turn Britain into a temple of high technology. At worst, while accidental leaks of radioactive materials continue, fears will persist that some future mishap will expose large numbers of people to potentially damaging doses of radiation. So it is also proper to acknowledge that, so far, the Sellafield reprocessing plant has not been a significant hazard to public life and health. In part, the reputation of the plant has been sullied by its proximity to the Windscale plutonium-producing reactor, where a fire in 1957 caused the world's most serious release of artificial radioactivity until the Chernobyl accident on 26 April this year. By comparison, the effects of Sellafield's string of mishaps are trivially unimportant.

Safety

Britain's nuclear inspectors conclude that a plant whose safety should be unquestioned cannot be allowed regularly to advertise its frailty. But may not the cumulative effect of Windscale's transgressions be more serious? That is the question that environmentalists are forever asking. There are three respects in which the question is apt and deserves a patient answer. The government of the Republic of Ireland is probably right to complain that the Irish Sea is more heavily contaminated with radioactivity than any other stretch of the world's continental shelves, but BNFL is probably also right to say that the contamination of human food chains is within acceptable limits; even though the discharge is now to stop, continued monitoring will be needed for decades to come. The occurrence of clusters of childhood leukaemia in villages near the plant is another puzzle;

a report by Sir Douglas Black two years ago may have correctly concluded that the clusters could be statistical fluctuations, but a diligent search for more immediate causes is plainly necessary. Finally, while Sellafield has a good record of keeping individual workers' radiation doses within statutory limits, the annual collective dose to Sellafield workers is uncomfortably large.

What else is there to complain about? The inspectors inevitably and, up to a point, fairly direct their chief criticisms at BNFL and its managers at Sellafield. But this is where the complications begin. In the old days the operators of the plant enjoyed a reputation for needless secrecy excused, but never fully justified, by the uses to which their chief product, plutonium, may be put. They were also in those days complacent, believing themselves to be fully in charge of a technically advanced plant (which was also true). But the inspectors underestimate the traumatic effects of the period, in the early 1970s, when the stuffing was knocked out of the British nuclear industry by the coincidence of several misfortunes, chiefly government disenchantment with nuclear power (born of the penury in which British governments have rubbed along ever since, but strengthened by the industry's over-optimism about its then new advanced gas-cooled reactor) and growing public hostility. The most damaging complaint in the report is that the present managers at Sellafield have taken to substituting formal approvals by advisory committees for their own judgements, thereby giving bureaucratic processes precedence over practical solutions for real problems. The old arrogance has given way to timorousness, no better suited to the operation of a potentially dangerous plant.

Future

In the present demoralized state of British public life in which nothing much is thought possible, the prospects are not bright for the year that BNFL has been given in which to put Sellafield in order. No doubt the company can fire a few managers and hire some others, but there is little hope of being able physically to rid Sellafield of its huge inventories of unwanted fission products and contaminated material until another public agency called NIREX (Nuclear Industry Radioactive Waste Executive) can negotiate a few sites at which waste materials might be safely stored. BNFL plans to start vitrifying highly active wastes roughly a year from now, but has nowhere other than Sellafield to house the products. And some of the unused facilities that clutter the overcrowded site cannot be dismantled because they are safer as intact relics than if they were broken into smaller pieces. Can NIREX, after five years of failing to find disposal sites, work magic in time to let BNFL complete its assignment?

The inspectors also complain that BNFL has been concerned with the short-term tactics of survival to the exclusion of strategic planning, which may be another consequence of Sellafield's recurring mishaps: BNFL's board members have been made into public relations men (which is not usually their talent). But much of the trouble is external to the British nuclear industry. In 1975, when BNFL had planned a new uranium oxide reprocessing plant at overcrowded Sellafield, it believed it could not follow the sensible course of putting the plant on a second site



for fear of a bruising public inquiry and, possibly, refusal. Ironically, it was forced to suffer the inquiry anyway. In present circumstances, the radical strategy of abandoning the old reprocessing plant, replacing it with another, might be safer and even cheaper, but the political obstacles to such a course would be formidable if not insurmountable.

So who is really to blame? The oscillation of successive British governments' policies on nuclear power are largely responsible for the lack of timely investment at Sellafield. The present government, while mildly supportive of nuclear energy in the sense of being prepared to back plans for building new reactors has shrunk from its more onerous responsibility, that of winning by persuasion a civilized accommodation with those dissenting from its own policies, which is the hallmark of good government. But what government would take up cudgels on behalf of an organization as accident-prone as BNFL's Sellafield? In the long run, BNFL's most serious failing may be none of those listed by the inspectors last week, but that of having provided the means by which British nuclear power has been made a formal political issue, with the Labour opposition party committed to the 'phasing out' of nuclear energy. □

Slogans for recovery

The United States must improve its industrial performance in its schools and colleges.

If the Iranian arms scandal blows over soon, some attention may be given in Washington to the emergent slogan of competitiveness, the means by which the US government and its advisers are seeking to accelerate the changing pattern of trade between the United States and the rest of the world. The motives are clear and even admirable. In spite of the devaluation of the US dollar relative to the yen and the deutschemark during the past year, the US trade deficit has not shrunk but, rather, grown to a larger amount than ever, \$37,700 million in the quarter ending in September, nearly 6 per cent more than in the previous three months. Although the volume of imports to the United States has indeed shrunk, higher prices (in dollars) of imported goods have kept the trade deficit growing. So it makes sense that the authorities should urge US industry to be more competitive.

Will the new bout of exhortation be more effective than the great fuss about declining US productivity three years ago? There are some obvious snags. In the fields in which US pride has recently been most humbled, motor cars and microchips, there is no reason to believe that US manufacturers invest less in capital equipment and automation than their more successful Japanese competitors. Nor is the United States short of the capacity for engineering innovation. The defects are more subtle — and thus will take longer to repair. The history of General Motors in the past few months is an illustration of how the managements of corporations can become detached from the products they make and from their ultimate customers. And while most of the new ground in microcircuit design has been broken by US companies, the constant worry about quality (or reproducibility) owes much to the shortage of skill on the production lines. Then the development of new products in typical US corporations has been bureaucratized to a degree that must delight the Japanese.

This is why the grave committees now being assembled to brood about competitiveness are certain to conclude that one ingredient of any long-term cure must be a means of persuading many more skilled people into manufacturing industry. Countries such as Britain have long sought solutions to this problem, but without success so far. But even if everybody knows what needs to be done, and has the will to do it, radical improvements of the educational system take decades, not months. Yet the only short-term solutions are crude and unpalatable — reductions of living standards. That is what the accumulations of trade deficits will, in the end, dictate. □

Solstitial considerations

The disappointments of 1986 have been balanced by good tidings of many kinds.

BEFORE readers of this and other periodicals lapse into the torpor of what seems increasingly to be an international holiday, with elevator muzak playing Christmas carols even in Japan, 1986 should not be mistaken for an entirely disappointing year. True, many of the disappointments have been cruel — Chernobyl, for example. AIDS came to seem more serious a threat to public health than had been thought. The summit meeting meant merely as a preparation for a summit raised peoples' hopes for arms control and then dashed them. The pace of economic growth has been disappointing, especially after the fall of the price of oil (which now seems to be on the way up again). Most countries in the West became more despondent about the difficulties of sufficiently educating the young. There was more than the usual harvest of discovered scientific fraud. Although Orlov was able to leave his Siberian labour camp, his comrade Anatoli Marchenko died in prison only a week ago. The apparatus of the US presidency has once again behaved as if above the law, and seems prepared to trade for hostages as well.

If the good news is, as always, harder to find; it is not less important on that account. People make their own tunes when whistling to keep up their spirits; for some, it is sufficient that there has been no final cataclysm, but that is too little to ask of a dead year, especially one in which there were cheerful signs of beneficial developments. Researchers, pilloried as they often are as denizens of ivory towers, may take particular pride at the speed at which some laboratory studies have been turned to practical ends. Chernobyl, for example, was heroically contained and has been understood. The fear that a large proportion of those infected by the AIDS virus may develop the disease has been partly offset by the hope that some kind of prophylaxis will emerge from the energetic attempts to develop a vaccine.

When it seems that changes of atmospheric ozone in the Antarctic spring may be sensitive indicators of anthropogenic threats to the ozone layer as a whole, the US Navy and the National Science Foundation are able to carry out speedy measurements, suggesting that growing fears of global change may at least be matched by improvements of monitoring capacity (and of understanding). Parallel computers have at last come of age (or will do so when programs have been written for them) while there is new generation of semiconductor devices, formed from GaAs by epitaxial growth, waiting in the wings.

In basic science, 1986 has been distinguished by its ingenuity, typified by the scanning electron microscope (which belongs to an earlier year, but whose inventors were awarded a Nobel prize in that now finishing). The argument (which may be wrong) that a 60-year-old set of gravity measurements contains evidence of a previously unknown force, in the same vein, only helps to show what should never have been forgotten, that the big battalions manning the accelerators do not have all the ammunition. The quasi-crystal saga has provoked many new lines of inquiry into the true structure of solids, while chemists must seriously think again about the electronic structure of molecules with conjugated bonds. The *in vitro* manipulation of plant and animal genes by people is now exquisitely delicate, but the real-life manipulation of genes by protein regulators has begun to be understood. It is no surprise that all this (and much more) should have happened in an otherwise unspectacular year; the surprise is merely that it has happened at a time when budgets have generally been cut, which may say something important about the response of researchers to adversity. The pity is that all this excitement has not been adequately communicated to those who ultimately, through taxes, pay the bills, as well as to their children. For 1986 seems also to have been the year for realizing that too few young people are being adequately trained to keep all these torches alight. □

Nuclear reprocessing

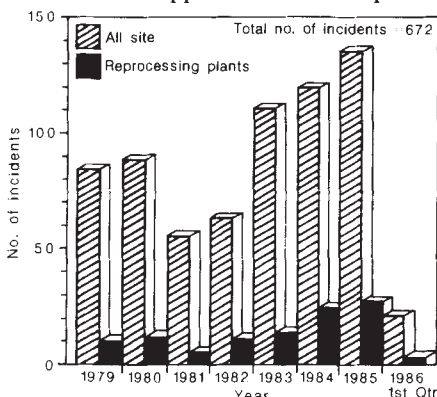
Sellafield threatened with closure within a year

THE management of Britain's only nuclear reprocessing plant, at Sellafield in Cumbria, has been given a year to improve the safety of the site or face closure.

The stern warning has been issued by the Health and Safety Executive (HSE), the licensing authority for the site, after an audit which it describes as "one of the most thoroughgoing physical examinations ever carried out in the UK at one time on any complex plant". The management of the complex, now operated by British Nuclear Fuels Limited (BNFL), is under no illusions about the seriousness of the findings, says HSE.

According to Mr John Rimington, HSE's director general, last week: "Our findings are not recommendations; they will have to be implemented. They match the comprehensive nature of the review. We intend that they should be carried out without delay".

HSE says that the BNFL management has attempted to improve the safety of the processing plant, which is on the site of Britain's oldest nuclear power station. But since 1979, there have been 672 'incidents' at Sellafield. The improvements have not been enough, concludes HSE. According to Rimington: "The main policies and priorities pursued by BNFL in improving the plant seem to us to have been the right ones. But the application of these priori-



ties has reduced the attention paid to potentially hazardous parts of the older plant. Some of these do not yet come up to the scrupulously high standard we demand of the nuclear industry."

One of the most 'hazardous' parts of the plant worrying HSE is the central area/building which performs the initial reprocessing, B205. The executive intends to put political pressure on BNFL to conform and Sellafield's operating licence will be amended to ensure that the improvements are implemented.

HSE gave particular attention to the

five incidents this year which caused disquiet and, in part, provoked the audit:

- 23/24 January — discharge of uranium into the sea;
- 5 February — leak from the central reprocessing complex B205;
- 13 February — fire on site;
- 18 February — pipe leak;
- 1 March — spillage of plutonium fuel pellets and subsequent contamination of contact surfaces.

The reprocessing complex at Sellafield is the home for more than 90 per cent of the radioactive waste produced by non-military power generation in the past 30 years. There are 16 reactors in 11 Magnox nuclear power stations in the United Kingdom. The uranium fuel rods of these reactors, enclosed in magnesium alloy containers or cans (Magnox), are stored in cooling ponds at the power stations before transportation to Sellafield; both rods and containers are subject to corrosion.

Chernobyl

Polish fallout underestimated

POLAND's official report on the Chernobyl disaster, published in a limited edition a few weeks ago, makes no mention of the consistent under-reporting of radioactive iodine contamination during the first few weeks after the accident. Nor has the International Atomic Energy Agency (IAEA) or the World Health Organization (WHO) been notified of the error, which is therefore perpetuated by the fallout maps produced by those organizations. The story has, however, been reported by several underground bulletins, notably Wokopach, the newsletter of pro-Solidarity nuclear scientists. The initial error was a genuine one. The emergency on 29 April, was originally set up in the 1960s to deal with the aftermath of a possible nuclear war, and its outdated procedures for iodine estimation systematically lost up to 75 per cent of the iodine before a reading was obtained. Normally, only a few of the 150 monitoring points operated by the public health service are working at any one time, and as the radio-iodine level was normally close to zero, the procedural error was not observed.

Early in May, scientists carrying out independent tests of fallout noted that their readings were consistently 3–4 times higher than the official figures notified to IAEA. The only exception was the official reading from Katowice, where the public health scientists had access to a new,

sophisticated estimation apparatus from the United States. At the end of May, the public health network notified the error to the special commission dealing with the effects of Chernobyl in Poland.

On learning of the error, the commission decided not to make it public. It justified this decision to its scientific consultants by pointing out that, a month after the accident, the worst danger from radioactive iodine was over (at that time, the commission did not know that emissions from Chernobyl had continued until 6 May), and that to reveal the error would spread unnecessary public alarm. The danger-level for milk had been set at 1,000 Bq, corresponding, at worst, to a true value of 4,000 Bq — and the level set for Vienna was 5,000 Bq.

Whether the commission was justified in keeping the information from the Polish public is a moot point. Withholding the information from IAEA and WHO, however, is much less excusable as both these bodies would surely have appreciated the commission's evident embarrassment over the error and the fear that its revelation might cause panic. Even less understandable is that the story of the error was suppressed in the official report, a publication for official distribution only. *Nature's* attention was first drawn to the matter by a government press spokesman, Mr Jerzy Urban.

Vera Rich



European Communities

Framework programme in limbo

DESPITE arguing until 4 a.m. on Wednesday last week, Europe's science ministers have failed to agree on a budget for the European Commission's 'framework' programme of research and development, a programme that aims to lay the basis for European cooperation in key areas of research and technology up to 1991. The ministers decided only one thing; to postpone a decision on financing until 22 December, when they will meet again in the hope of completing a compromise which began to emerge in the small hours last Wednesday.

However, the new deal, which involves a three-year rather than five-year programme, has a long way to go before it can form the basis of an agreement. The Commission had tabled a 7,735-million-ECU (£5,400-million), five-year programme, but last week the British, French and West German delegations were sticking on low figures of 3,500–4,000 million ECU, the Dutch on 6,000 million and the other eight delegations on the full amount proposed by the Commission. The gap between the extremes was thus more than a factor of two, and it was difficult to see last week how it could be bridged by the Commission's compromise proposal, of 3,700 million ECU to be committed, and 3,100 million ECU to be spent. These sums would cover three years not five.

Also against the new proposal is the European Parliament, whose energy, research and technology committee has condemned any reduction in the programme, and urged the Commission to prepare to withdraw the whole proposal. Jacques Delors, the president of the Commission, in reply to an address by the British Prime Minister, Mrs Margaret Thatcher to the parliament in Strasbourg last Tuesday, warned that he had instructed research commissioner Karl-Heinz Narjes to abandon the framework programme if ministers failed to give it a reasonable level of financing. Thatcher had just addressed the parliamentarians on the six months of British leadership of the European Communities (as president of the decision-making Council of Ministers) without making any serious mention of research. Delors gives the framework programme a high priority, and was clearly angered that Thatcher had proved an obstacle to its implementation. However, within a few hours, Narjes was proposing the new compromise. Parliamentarians have since expressed their dismay at this outcome, and are pressing for a thoroughgoing confrontation between the Commission and the Council of Ministers.

Finally, however, it may be a smaller straw that breaks the Commission's back: the matter of a 12.6-million ECU increase

in the 700-million-ECU four-year budget of the Joint Research Centre's, the group of four laboratories in Italy (Ispra), West Germany (Karlsruhe), Belgium (Geel) and the Netherlands (Petten) which are wholly funded by the Commission. Last week the research ministers received a critical report on the centres prepared by an independent group of European industrialists, and although the Commission already has plans to travel down the road the industrialists recommended (greater interaction with industry, better management of funds), most ministers were sufficiently confirmed in their negative view of the centres that they refused any increase. The exception was the Italian minister, who benefits from the considerable research employment that the largest of the laboratories, Ispra, provides.

Embryo research

UK legislation further delayed

STILL wriggling uncomfortably on the hook of human embryo research, the UK government last week issued a consultation paper on *Legislation on Human Fertility Services and Embryo Research* (Cm 46, HMSO, £2.90), asking for responses by the middle of 1987. The aim is then to introduce legislation, although it seems likely that a general election will intervene.

The paper is based on the Warnock report, published in 1984, since when the government has avoided three attempts by members of parliament (MPs) to prohibit almost all human embryo research, while promising its own legislation. From these attempts and other responses, the paper divides Warnock's recommendations into those where "a broad measure of agreement is likely" and those where it is not.

Chief among the contentious issues, according to the paper, is the use of human embryos for research purposes. One camp argues that research is both necessary and warranted. It is necessary to improve infertility treatments, contraceptive techniques and the detection of early abnormalities. And it is warranted for 14 days after fertilization. The other camp is opposed to any research that leads to the destruction of an embryo because this "is tantamount to murder", says the paper.

Pointing out that even the members of the Warnock committee were split on this issue — as are government ministers — and that various intermediate positions have been expressed, the paper says that the government intends to offer parliament alternative sets of draft clauses dealing with the issue, and to allow MPs to vote according to their consciences rather than along party political lines.

Previously fully in support of the framework programme, the Italian delegation has now linked its support to agreement on the increase for the research centres. This must make considerably less likely the unanimous agreement that is necessary for the framework programme to go forward, and it seems probable, barring a miracle, that the framework programme will limp forward, unagreed, to the next six months' Belgian presidency of the Council of Ministers. This would leave a number of programmes hanging loose, such as ESPRIT in information technology, RACE on the development of Europe-wide integrated broad-band communications, EURAM on advanced materials and the unstarted joint research programme on AIDS. There is around 1,050 million ECU in the European kitty next year to continue existing programmes, but this is not enough to increase the pace of cooperation intended.

Robert Walgate

Other issues that are considered contentious are the possibility of egg or embryo donation and whether human sperm should ever be allowed to fertilize animal eggs, even as a test for sperm quality with destruction of the embryo after one division.

Although commercial surrogacy was made illegal in 1985, the paper invited comment on whether the law should be amended to allow the surrogate mother and the commissioning parents to be prosecuted. It also seeks views on the possibility of a non-commercial surrogacy service.

Only one reaction to the consultation paper is unanimous, namely that it is a delaying tactic. Mrs Phyllis Bowman, director of the Society for the Protection of Unborn Children, says the paper is nothing but a rehash of the Warnock report. A spokeswoman for Progress, a lobbying group in favour of human embryo research agrees but sees the delay swinging public opinion in the group's favour.

The last attempt to prohibit human embryo research was on 13 October when Mr Ken Hargreaves MP introduced legislation in a way that stood no chance of being accepted but at least forced a vote. As on past occasions there was a substantial, if reduced, majority in favour of the proposed bill. Meanwhile, such research continues under the auspices of the Voluntary Licensing Authority set up last year by the Medical Research Council and the Royal College of Obstetricians and Gynaecologists. The authority tends to follow the recommendations of the Warnock report, which are sometimes more liberal than local ethics committees through which medical research proposals have also to pass.

Peter Newmark

Medical research

Backs to the wall in Britain

THE British Medical Research Council (MRC) faces a deficit of £13.2 million by the end of the decade even if it does no more than maintain the existing rate of awards and renew long-term grants at an appropriate level. But there is also a pressing need to spend more on research into AIDS (acquired immune deficiency syndrome) and to offer more competitive salaries to keep the best scientists, says Sir James Gowans, secretary of MRC.

Introducing the council's annual report in the year before he retires, Gowans described AIDS as a "national emergency" that deserves special earmarked research funds. Discussions with government departments seem likely to produce the £0.5 million needed immediately for one major and two minor AIDS projects, but several million pounds will be needed over the next few years. (A bare £2 million was spent on AIDS research by MRC and the Department of Health and Social Services in the past year compared with the \$40 million predicted for 1987 in the United States.) Priority must be given to monitoring the spread of the virus that causes AIDS and the effect of the campaign to reduce the spread, said Dr D. A. J. Tyrrell, chairman of the MRC working party on AIDS. But neither he nor Gowans would be drawn on how to overcome the problems that are holding up monitoring (see *Nature* 324, 396; 1986).

On salaries, Gowans expressed frustration that MRC's grant-in-aid, worth £129 million last year, assumes an increase of only 3 per cent this year and 2.5 per cent next year when pay awards are running at 5–6 per cent. The level of awards for its staff is not controlled by MRC as it is tied to increases for their university, clinical and civil service equivalents. That, and the decreasing budget, also constrains the ability of MRC to offer salaries that compete with offers from industry and overseas. At the top level, however, the council is free to offer more competitive salaries as long as the mean is maintained at the average for professors in universities, which have the same flexibility.

Unless the MRC budget is increased, said Gowans, it will be extremely difficult for the council to grasp new opportunities in research. The problem is already very serious; 90 project grants were approved but not funded in the past financial year. Royalty income from discoveries made by MRC scientists is expected to increase in coming years, particularly through the activities of the new Centre for Collaborative Research. But last year it was only £0.1 million and a limit of £2.5 million has been imposed.

Peter Newmark

Japanese volcanoes

Forecasters wise after the event

Tokyo

JAPAN'S Volcanic Prediction Liaison Council seems to have been rewarded for its failure to forecast the behaviour of Mount Mihara on Oshima, the volcanic island south of Tokyo evacuated during a major eruption last month. The council has won substantial new facilities on the island that will make Mihara one of the best-monitored volcanoes in the world.

The council, advisor to the Meteorological Agency, has come under fire for its pronouncements about Mount Mihara. At the end of October, following the outbreak of volcanic tremors, the council declared that any eruption would be "several months or years" away. Even when the

graded before the islanders return.

The number of seismometers on the island will be almost doubled, to nineteen. Seventeen new tiltmeters will be put in place along with various other pieces of equipment costing a total of Y1,100 million (£5 million). The seismometers will be linked to telephone lines and 40 engineers of Nippon Telephone and Telegraph are racing to link the seismometers to a telemetric network by 19 December.

Preliminary analysis of the November data suggests that prediction will still be unreliable, and that details will often be discovered only after the event. Seismograph data did, however, play an important role in the evacuation, according to



The Mount Mihara eruption at its peak on the night of 21 November.

lava started spewing out of the volcano two weeks later, it was confidently predicted that the flows would be confined to the caldera. But when a new line of vents broke through the caldera rim, sending lava surging towards the main town Motomachi, and the island had to be evacuated, the council turned about and started issuing warnings that mixing of sea water and magma could lead to a phreatomagmatic eruption, as at Krakatoa.

Now, almost a month later, with the volcano silent and the government running up huge bills to support the evacuees, the council has been under pressure to issue an all-clear. Finally, last Friday, its members adopted a "unified view" that Mount Mihara is now calm "in the short term"; the Tokyo Metropolitan Government immediately announced that the islanders would be allowed to return to Oshima between 18 and 22 December. But the council's decision was given on the precondition that the island's volcano-monitoring network be substantially up-

Professor Aramaki of Tokyo University Earthquake Research Institute, who spent the night of 21 November at a police station in Motomachi. A shift in the earthquake swarms to a position off the south-east coast of Oshima, reported by telephone to Aramaki by researchers at Tokyo University's observatory on the island, coincided with police reports of new cracks in a road near Habu, the south-east port of Oshima; acting under Aramaki's advice, police removed evacuees from Habu to the northern ports of the island.

The new equipment will be operated by Tokyo University and the Meteorological Agency as well as various other government agencies. But it remains to be seen whether they can work together. While Tokyo University researchers stuck to their posts on 21 November, as a river of lava bore down on their observatory, officials of the nearby Meteorological Agency Observatory were among the first to flee the island. Feelings between the two groups are strained.

David Swinbanks

Pesticides

FDA told to try harder

Washington

THE US authorities are not doing enough to catch and prosecute unscrupulous food growers who may be steadily poisoning the American public through illicit use of pesticides. A study* by the General Accounting Office (GAO) concludes that the US Food and Drug Administration (FDA) is the agency most at fault.

The study shows that the proportion of tested domestically produced food is small (probably less than one per cent), and, more significantly, that over half of pesticides that may be present are not detected by any of FDA's five preferred 'multi-residue' tests. Eight out of the nine most dangerous pesticides used are not detected by these standard tests. The agency uses the more time-consuming and expensive single-residue tests only when it has reason to suspect chicanery.

The proportion of tested samples has fluctuated since 1979 between 1.8 and 4.5 per cent, but more than 5 per cent of fish samples tested over the same period contained illegal residues. FDA says these proportions are not representative of the food supply as a whole, as trouble spots are sampled more often.

But the most disturbing discovery of the report is FDA's record at preventing the sale of adulterated food and prosecuting wrongdoers. In 107 out of 179 cases in which the agency found illegal residues (over a period of more than two years), the food had already been sold by the time the result came through, and in none of the 179 cases did FDA seek criminal penalties. Because the agency lacks detention authority over domestic food, it often cannot prevent such food being exported—but it can seize adulterated imports.

FDA also lacks legal authority to impose civil penalties, a shortcoming GAO would like remedied to provide an effective deterrent. The alternative, costly criminal procedures, is rarely used. Congress may hold hearings on the agency's effectiveness at pesticide control next year, and should be interested in the idea.

Does all this mean that Americans are being poisoned? Not quite. Illegal residues may simply be pesticides applied to the wrong crop, or may exceed approved 'tolerances' by a small amount. The tolerances are supposed to have large built-in safety margins. Environmental groups say, however, that the factor of 10 sometimes used in extrapolating safe dosages from animal studies is inadequate and that tolerances are set before all the relevant data are collated.

Tim Beardsley

Pesticides: Need to enhance FDA's ability to protect the public from illegal residues. (GAO, 1986).

UK industrial research

More stick than carrot on offer

BRITISH manufacturing industry, part of which enjoys lucrative defence contracts, must itself spend more on research and development, says Mr John Fairclough, the government's chief scientific adviser.

Annual government investment in research and development is £4,300 million, of which 51 per cent is channelled into the Ministry of Defence (MOD). In sharp contrast, the Department of Trade and Industry (DTI), one of the principal sources of industrial research funds, has seen its share fall from 7.9 per cent of the pool in 1983-84 to an estimated 5.2 per cent in 1988-89.

The call for more investment came on the publication of the government's review of research and development expenditure. Although Britain invests more in publicly funded research and development than most of its European neighbours, but as a proportion of Gross Domestic Product, the total devoted to civil projects lags behind West Germany, France, Japan and the United States.

Fairclough, who began a two-year secondment from IBM (UK) to the Cabinet Office in May, is attempting to change that ratio. One solution is for companies publicly to disclose their research and development expenditure. Such disclosure, Fairclough believes, would make financiers aware of the long-term strategies being adopted by companies.

The Fairclough solution was not the only one to emerge from Whitehall last week as the government attempted to forge bet-

ity and industry. Government funding is spread over five years and is expected to attract the same from industry.

Mrs Thatcher, in announcing the programme, said that "the excellence of British science has not been matched by a rapid pace of application by British industry of the newly emerging technologies. Nor is the private sector investing as much



Fairclough — more for research.

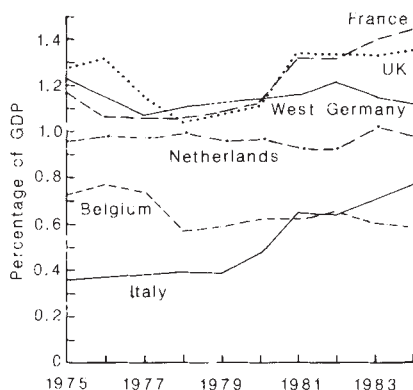
of its own resources in research and development in this country as are our more successful competitors."

The success of the LINK programme will require cooperation between government departments which in the past have been noted for their rivalry. They are the departments that already have research budgets — the Departments of Trade and Industry, Education and Science, Energy, Environment, Transport, Health and Social Security, Agriculture Fisheries and Food and the Ministry of Defence. The Scottish, Welsh and Northern Ireland offices will also be involved.

There will be a steering committee composed of senior industrialists, academics and officials from the relevant departments and research councils with a brief to foster strategic areas of scientific research directed towards "the development of innovative products, processes and services by industry"; to stimulate industry to increase its investment in research and development; to encourage industry to exploit developments in science; to make scientists more aware of the needs of industry; and to develop technologies that cross the normal boundaries of scientific disciplines.

The Advisory Council for Applied Research and Development, the Advisory Board for the Research Councils, the Assessment Office on Science and Technology in the Cabinet Office are collaborating to find other ways of marrying research to industry.

Bill Johnstone



ter links between its departments, the universities and industry.

Another, credited to the Prime Minister, Mrs Margaret Thatcher, is a programme for re-routing £210 million of the budget for scientific research and development to "speed up the development of products and services from scientific and technological research carried out by universities, government departments and industry".

The programme, called LINK, is to encourage research and development partnerships between the scientific commun-

Nuclear safety

United States opts for caution

Washington

THE principal US military plutonium production reactor, a graphite-moderated plant similar to that at Chernobyl, is to be shut down for six months by the Department of Energy (DoE) to carry out urgent safety modifications which could cost about \$50 million.

The 23-year-old 'N' reactor on the Hanford military nuclear reservation in Washington state has been a focus of concern since the Chernobyl accident. Besides being graphite-moderated, like Chernobyl-4 it lacks a containment structure capable of withstanding steam and



N reactor at Hanford site.

hydrogen explosions that might occur in an accident. Two out of six independent consultants, asked by DoE to assess N-reactor safety, recommended closing down the reactor immediately and permanently, unless to do so would threaten national security.

Others said the poor containment meant the consequences of an accident would be worse than at a commercial power station, and that they could support continuing operations only if further safety checks were made. DoE declined the invitation to shut down the reactor permanently, but has accepted the need to spend immediately \$50 million on improvements.

The consultants, under Louis H. Roddis, submitted independent reports, agreed that there was virtually no possibility of a Chernobyl-like accident, but found that radioactivity might be released following a minor accident because of doubts about the N reactor's 'confinement' system, which is designed to lower pressure in the reactor building after an accident by spraying cold water. The system has never been tested under realistic conditions. One consultant commented that the list of required improvements is so extensive that a commercial reactor would be required to shut down to carry them out. Management improvements were also urged.

The consultants urgently recommended installing a hydrogen mitigation system, and a 'basin' for collecting radioactive wa-

ter that would be released in an accident. Hydrogen explosions figured in both the Chernobyl and Three Mile Island accidents, but the N reactor confinement system would not withstand an explosion of the size thought possible, according to the consultants. And under present plans, contaminated water would be temporarily stored in a desert pit, a plan DoE acknowledges to be inadequate. The department says it will start to install a hydrogen mitigation system immediately and also accelerate a comprehensive probabilistic risk assessment now in progress.

While announcing the temporary shutdown, DoE also said it was awarding a 5-year, \$4,000-million contract to manage the entire Hanford operation to Westinghouse. The N reactor was previously man-

aged for DoE by the General Electric Company, and some of DoE consultants noted "complacency" over safety. Another contractor at Hanford, Rockwell International, has been the focus of allegations of poor management at two plutonium production plants recently shut down temporarily for safety violations.

Whatever safety improvements are made, the N reactor will not be in business for much longer. Commissioned in 1963 with a design lifetime of 20 years, its pile is now expanding at the rate of 0.8 inches per year, putting stress on coolant pipes and threatening to hit the thermal shield in as little as 3 years. That will be the end for the reactor as replacement of the whole pipe is not feasible. DoE is now trying to decide whether it needs another plutonium production reactor (it has others at the Savannah River complex in South Carolina) for next century's nuclear weapons.

Tim Beardsley

Albanian outlook

Changing tack on exports

RAMIZ Alia, who became leader of the Albania Workers' Party (AWP) on the death last year of Enver Hoxha, has an unusual approach to his country's non-renewable natural resources. "The interests of future generations", he told the AWP congress last month, demand that such assets as copper and chromite ores be "put into economic circulation as soon as possible", by extracting and exporting them. "It is better to leave these natural assets to future generations in the form of factories, plants and combines, as means of production, as functioning assets, rather than in the form of potential resources hidden underground", he said.

Although he was referring chiefly to mineral ores, Alia's speech made it clear that Albania's new five-year plan will attempt also to increase its capacity for exporting oil and coal. Albania, the least developed country in Europe, thus differs markedly from most developing countries which see the massive export of raw materials as a vestige of colonialism, and aim instead at the export of finished or semi-finished products with a larger added value. But the policy of Albanian self-sufficiency established by Hoxha after his breaks with the Soviet Union and then with China means that the country must implement its plans for modernization without foreign loans or grants-in-aid.

In these circumstances, Alia's view that future generations can best be served by selling off the country's irreplaceable assets does make some kind of short-term sense. Albania's economy is still based largely on human muscle-power. The 'prestige' civil engineering projects of recent years, such as hydroelectric dams and railways, have largely been built by

student volunteers, equipped with little more than pick and shovel. Agriculture is labour-intensive, and Alia's promise to "increase the role of science in its service" will entail not a decrease in the numbers employed but a "considerable increase".

Science in the service of production is one of the main targets of Alia's programme. This policy demands trained personnel, in a country where at present only 57 per cent of young people receive more than primary education. (By 1990, according to the plan, this figure should rise to 73 per cent.) Alia's speech stressed not only the need for more trained scientists and technicians, but also for better management of science. The Albanian Academy of Sciences, he said, must give "more initiative and thought" to the directions in which the sciences should develop, while the Committee for Science and Technology must provide "better planning, a clearer definition of priorities and more effective organization and coordination of manpower and resources". The University of Tirana and other institutions of higher education must become "bodies of consultation for the broad masses of specialists in production", while the ministries concerned must refrain from turning educational and research centres into mere "administrative apparatuses".

In spite of Alia's boast that Albania can now train all the specialists it needs, and in spite of "self-sufficiency", the country is still, of course, largely dependent on foreign scientific achievements. And to enable Albanian scientists and technicians to make use of foreign results, the Ministry of Education has been instructed to remedy the "unsatisfactory situation" in the teaching of foreign languages. Vera Rich

Australia

Lay head for CSIRO board

Sydney

THE initial reaction of the Australian scientific community to the appointment of former New South Wales Premier and federal president of the Australian Labour Party (ALP), Mr Neville Wran, as head of Australia's largest research body, the Commonwealth Scientific and Indust-

rial Research Organization (CSIRO), lay somewhere between surprise and shock. The parliamentary opposition described the appointment as "appalling political cronyism". Many CSIRO scientists were dismayed by Wran's appointment, which marks the end of a 60-year tradition of having a scientist at the head of CSIRO,

but this feeling seems to have given way to guarded optimism that Wran's undoubted political acumen and extensive connections with industry will be great assets.

Wran was premier of New South Wales for 10 years and federal president of ALP for 6 until his surprise resignation from both positions last July. His government would not have had to face another election until early 1988. The stated reason for his resignation was that he was tired of politics and that stepping out mid-term would give his successor time to get used to the reins of government before having to face an election.

Wran's appointment coincides with the disclosure of legislation designed to give CSIRO a new board and structure. The changes are the result of recommendations by an Australian Science and Technology Council review of the organization's operation. In the new board, policy-making and management functions have been separated to make it more like the board of a normal corporate body. The old board consisted of three full-time members, all distinguished scientists, and five part-time outside members. One of the full-time members was chairman and chief executive, a position held in the last board by Dr Keith Boardman. In the new model these posts have been split, with the chief executive becoming the only full-time member of a 10-man board. The chief executive is thus the most senior CSIRO scientist. Boardman has been appointed permanently as chief executive subject to the approval of the board which is due to meet for the first time this month.

It will be the task of the new board to align CSIRO's research ambitions with national priorities, which means increasing emphasis on industrially applicable research, particularly on that applicable to manufacturing, although Boardman is careful to point out that this does not mean a diminution in either the quantity or quality of fundamental and rural research carried out by CSIRO. In fact, the number of scientists working in rural research has increased because of more funding from outside sources despite the decreases in government money.

In addition to Wran, the part-time board members include two scientists, a farmer, a representative from the union movement and four representatives from industry, so it should be well suited to its task of linking CSIRO to the strategic needs of industry.

The Minister for Science, Mr Barry Jones, has long bemoaned the poor record of scientists for standing up in public and speaking out for their own cause, and has even gone so far as to call them "wimps". Perhaps the appointment of a politician to head CSIRO means that Jones has concluded that the best way to get the scientists' case heard is to give them a voice to speak for them.

Charles Morgan

Britain gets Cray supercomputer at last...

THE UK Science and Engineering Research Council (SERC) has at last taken delivery of its Cray X-MP/48 supercomputer, after much discussion between the British Department of Trade and Industry and the US Department of Commerce.

For months, the latter has been concerned that 'foreign aliens' might gain easy access to the supercomputer which is to be housed at the Rutherford Appleton Laboratory. The Americans wished to impose rigid restrictions on SERC, a move vigorously resisted by the British authorities.

For logistic reasons, trade officials from both sides of the Atlantic had been unable to meet to discuss the subject, so the Americans have approved the purchase to prevent any further delay but without receiving the assurances they originally sought. The trade authorities will meet soon to discuss the 'principles' of the case. The

machine will be operational by the end of January and will run by SERC on behalf of the research councils and the universities. SERC says the Cray "will be a considerable boost for UK scientists and engineers engaged in a wide range of computer simulation problems", including the physics of liquids and solids, large-scale-integration circuit design and aircraft design.

The supercomputer has been acquired partly as a result of a report prepared for the Advisory Board for Research Councils, the Computer Board and the University Grants Committee, entitled *Future Facilities for Advanced Research Computing*, by a working party under the chairmanship of Professor John Forty of the University of Stirling, who has agreed to be chairman of the management committee for the supercomputer service.

Bill Johnstone

...and Indian supercomputer is on the way

New Delhi

AFTER protracted negotiations, India and the United States have signed a document paving the way for the sale of a Cray-XMP supercomputer to India. The deal was approved in principle by the US administration eight months ago but became bogged down because of disagreements over the US terms and conditions for its sale. Indian demands that its sovereign rights should not be violated in any arrangement and US fears about possible misuse by India and potential leakage of technology to the Soviet bloc were the bone of contention. The problems were resolved and mutually acceptable conditions of safeguards were finalized last week after three days of intense negotiations between the US delegation led by Assistant Secretary of State Robert Dean and officials of India's External Affairs Ministry. The signing of the safeguards document has cleared the way for India to become the first non-NATO country to receive the computer.

According to a US spokesman, the safeguards are similar to those applicable to US computers sold in Britain and West Germany. The United States, which initially insisted on unrestricted right of inspection to check diversion of technology to

third countries, finally agreed to exercise the right only in the event of suspected diversion. It also wanted to station US personnel permanently at the computer facility, but agreed to the Indian request that US participation should be a temporary arrangement to be reviewed after 2-3 years when Indians would be ready to man the facility on their own.

The supercomputer, which is expected to cost about \$20 million, will be installed in New Delhi under the operational control of the Department of Science and Technology. It will be used for meteorology but the United States has agreed to let India use any spare capacity for certain stipulated other areas of research excluding nuclear or defence-related spheres.

The Indo-US deal on the supercomputer is being keenly watched by Japan. As negotiations with the United States were proving difficult, India had six months ago placed orders for three supercomputers from Japan, but the export was stopped under pressure from the United States. Now that the safeguards have been worked out, Japan will have no difficulty in exporting similar computers to India, which hopes to install four of them by next year.

K. S. Jayaraman

Rabies vaccine

Inquiry into Argentine trials

Washington

OUTRAGE and calls for investigations have followed in the wake of revelations about field trials of a recombinant rabies vaccine conducted by the Pan American Health Organization (PAHO) (see *Nature* 324, 202; 1986). Both the National Institutes of Health (NIH) and a congressional committee have begun inquiries into the affair, in particular into the role of the Wistar Institute of Philadelphia where the vaccine was made. Although the government of Argentina has made no formal comment on the tests, members of the Argentine scientific community feel the trial was conducted without safety precautions that would have been required had the trial been carried out in the United States (see p.610). But Hilary Koprowski, director of the Wistar Institute, says people should be rejoicing that the new vaccine seems to be a success and not complaining about an experiment now over.

The new vaccine uses a vaccinia virus genetically engineered to contain an immunogenic gene from a rabies virus. Tests on laboratory and captive animals have shown the vaccine to be successful in in-

ducing antibodies against the rabies virus. A total of 20 cows were vaccinated at the PAHO field station in Azul, 180 miles south of Buenos Aires. Ten cows were vaccinated intramuscularly and ten subcutaneously. Each group was kept in an open paddock with 10 unvaccinated cows.

But the field test, begun in July, was terminated in early fall after a commission of Argentine scientists visited the Azul station and raised questions about the way the experiment was being conducted. The commission reported that there were no signs announcing the nature of the experiment, both wild and domestic animals could come in contact with the vaccinated cows, excreta from the vaccinated animals was not being treated and milk from the vaccinated cows was being consumed by humans and animals at the Azul station. According to Mercedes Weissenbacher, one of the commission members, some of the milk was sent to a local dairy where it was used for making powdered milk and confectionery. Koprowski says the milk should have been frozen and sent to Wistar's French collaborator Rhone-Merieux to be tested for the presence of virus.

PAHO agreed to terminate the experiment after the commission's visit to Azul. Although Koprowski complains that PAHO was prevented from obtaining further blood or tissue samples from the vaccinated animals to see if the vaccine was still effective, Weissenbacher says blood and milk samples were collected before the animals were killed last month and are being stored for analysis.

Koprowski is upset that the animals were killed before they could be challenged with virus. He maintains that initial results were successful; the virus was not shed, neither humans nor animals became sick and the vaccinated cows developed antibodies to rabies virus.

PAHO has declined to answer questions on the trial. Carlyle Guerra de Macedo, director of PAHO, admits "mistakes were made in the way the matter was handled", but says a full disclosure will have to wait until discussions with the Argentine government are concluded.

Jose La Torre, director of the Centro de Virologia Animal and a member of the Commission sent to investigate conditions at Azul, says he agrees that the vaccine trial was an important one, but he objects to the way it was conducted. Weissenbacher concurs, adding animal care workers should not have been involved in the vaccine trial without obtaining permission from Argentine health officials. In justifying performing the experiment in Argentina, a PAHO spokesman said last month that cattle losses from rabies in Argentina amounted to millions of dollars a year. But a spokeswoman for the Argentine embassy in Washington said such losses were closer to \$60,000 a year.

La Torre visited Wistar in 1984 along with representatives of the Argentine pharmaceutical company Paul which was interested in trying the new rabies vaccine. Koprowski says Wistar told the Paul representative such a commercial arrangement would have to be made through Transgene, Wistar's French collaborators. Paul never contacted Transgene, but La Torre says that following his visit he wrote to officials at Wistar, informing them that they would need permission from the Ministries of Agriculture and Public Health to conduct a field trial. An Argentine law, adopted in 1903, prohibits the import of exotic viruses into Argentina. Wistar associate director Warren Cheston says a preliminary document from Wistar to Paul concerning a licence for the vaccine charged Paul with responsibility for notifying Argentine officials.

News of the Azul trial reached the Argentine scientific community when Mauricio Seigelchifer, an Argentine scientist visiting Wistar, wrote to colleagues about the experiment. Although Seigelchifer's contract with Wistar ran until the end of the year he returned to Argentina last week.

Joseph Palca

Israel

University shake-up on cards

Rehovot

A MAJOR reconstruction of the higher education system in Israel that would create a two-tier structure, halt the growth in the number of students at existing institutions and raise tuition fees has been proposed by Professor Jacob Ziv, head of the Grants and Allocation Committee of the Council for Higher Education. Ziv, who oversees the allocation of US\$246 million a year to the universities, believes there is no alternative if the growing demand for higher education is to be eased.

The universities' share of the national cake has substantially declined in the past decade. In the mid-1970s, they received 7.7 per cent of total government budget allocations (exclusive of defence expenditure and debt repayment); this year it was 4.4 per cent. This reflects a change in priorities, as during the same period government support of education (below university level) and of health services went up by nearly 40 per cent.

There has been a change for the better since the present government took office in 1984, but funds remain short and there is no solution in sight for the debts of \$200 million of Israeli institutions of higher education, about half of which are borne by the Hebrew University. They have become so onerous for the university that it delayed opening its school year and its pre-

sident and director general have resigned.

Although Ziv, formerly dean of electrical engineering and vice president of academic affairs at the Haifa Technion, wants more government support, he also favours restructuring higher education.

His most far-reaching proposal is for a two-tier system to halt the growth of the student population at present undergoing higher education (now increasing by about 3 per cent a year) and perhaps to reduce the numbers. Young people unable to qualify for admission under the new rigid standards would go to two-year study centres; if they did well, they could eventually transfer to a university. These centres would be inexpensive "teaching factories" staffed mainly by part-time lecturers who would not enjoy the conditions or research facilities of university personnel.

A ministerial committee is now studying Ziv's proposal to raise university fees (now about \$1,200 a year). But as he sees it, less affluent students should be allowed to pay their fees after graduation.

University libraries are less well stocked than they used to be, modern equipment is often lacking and salaries are only a quarter of those paid in the United States. The result has been a brain drain and even scientists who retain their posts in Israel often do much of their research overseas.

Nechemia Meyers

Wistar's export to Argentina

SIR—Argentinian scientists have recently been disturbed by an experiment involving both humans and domestic animals infected with a live recombinant vaccine. [An account of the experiment appeared in *Nature* (324, 202; 1986) after this letter was written.]

The experiment was carried out in an experimental farm of the Panamerican Health Organization of Azul, province of Buenos Aires, following a protocol designed by the Wistar Institute of Philadelphia; it was supervised by Dr J. Held (director of Centro Panamericano de Zoonosis, CEPANZO, PAHO), Ana Maria Diaz (head of the rabies unit), Elmo de la Vega (chief of pathology) and Luis Lazaro (chief of the Azul Field Station).

The experimental protocol was presented by Dr Roberto Weimann from the Wistar Institute, who flew to Buenos Aires immediately after the nature of the experiment became public, thanks to the action of a group of scientists from AADICYT, the Argentinian Association of Research Scientists. Soon after, the Argentinian Ministry of Health appointed a special committee of inquiry. After interviewing Weimann and visiting the premises of CEPANZO at Azul, the committee recommended the immediate interruption of the experiment, based on the following evidence:

(1) The experiment was started on 1 June 1986, without the permission, consent or notification of any Argentinian human or animal health authority.

(2) The effects and eventual risks of the inoculation of a rabies-vaccine recombinant virus in open-field cattle and its possible transmission to humans are completely unknown. The only reports in the current scientific literature deal with laboratory animals (mice and rabbits, *Proc. natn. Acad. Sci. U.S.A.* 81, 7194, 1984) and foxes kept in captivity (*Nature* 322, 373; 1986). There is particular concern about a possible alteration in the tissue localization of the recombinant virus as in the case of the Moloney murine leukaemia virus-rabies recombinant (M.P. Kriegler and C. Perez, Scientific Report 84/85, Institute for Cancer Research, Fox Chase Cancer Center) that might produce long-term consequences difficult at present to assess.

(3) There were serious violations of ethical, ecological and safety rules:

- The inoculated animals were not kept in isolation, permitting the free access of other wild and domestic animals to the area, with the danger of spreading the new virus in an open ecosystem.
- No warning signs were placed at the experiment area.
- There were precise instructions that the

same two animal handlers should milk each group of cows throughout the experiment.

- The four animal handlers were not vaccinated against smallpox before starting the experiment. The only sign of previous immunization considered by those responsible for the experiment was the presence of a scar compatible with vaccination.

- The four animal handlers were not under medical surveillance.

- Some of the milk from the inoculated cows was consumed by the animal handlers and their families without being pasteurized. The bulk of the milk was sent to the local market, and distributed to the community after pasteurization.

- The way in which this experiment was performed clearly infringes the World Health Organization's recommendations about experience involving recombinant DNA technology (Bethesda, 1984, Geneva, 1985).

We are not against the development of recombinant vaccine technology in view of its potential value for effective disease control. But we think that recombinant vaccines, like any other recombinant DNA project involving humans, must be developed in the light of international laws and recommendations, caring ethical and ecological consequences.

Moreover, we feel that our country has been illegally used as a test field for a kind of experiment that is not yet accepted in the countries where basic research on this vaccine had originated.

PABLO R. GRIGERA

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- This letter is signed by 134 Argentine scientists.

Defensive deterrent

SIR—At the time I was approached, I objected to the Cornell survey of National Academy members (*Nature* 324, 4; 1986) as to the feasibility and desirability of the Strategic Defense Initiative (SDI) even though I agree that no defensive system would be able, within the next 25 years, to provide effective population defence, even if the Soviets froze their arsenals at current levels. But as Reykjavik (which took place after the survey was completed) demonstrated, this is not the right question. Rather, we should be asking whether, if arms control leads to a mutually agreed reduction in the offensive threat to, say, a few hundred offensive missiles, can a defensive system be effective against this much lower threat? The answer to this question is probably yes; I would guess that many of my fellow academicians who

denied the feasibility of a defensive system against the current threat would concede that defence is feasible against a lower offensive threat. That those responsible for the Cornell survey did not give the respondents this choice I consider to be an improperly restrictive view of the matter.

Indeed, the entire episode reflects a curious bias in the academic world against the so-called defensive-dominated world. A defence-dominated world in which peace is maintained by denial of aggressive intent is surely morally superior to the present offence-dominated world in which peace is maintained by threat of mutual genocide — provided the defence-dominated world and the transition there-to can be adequately stable.

Rather than sanctifying the current offensive world as being the only one in which peace is maintained, academic peace studies groups ought to be examining in full seriousness the defensive world. Reykjavik gives us a glimmer of hope that the first step to a defensive transition — deep reductions in offence — is not a will-o'-the-wisp. How to stabilize such reduction by appropriate defensive deployment and how to redirect SDI towards this goal ought to be central elements in academics' peace agenda, much more than ill-considered, and I fear, politically-inspired, attempts to discredit any movement toward a defence-dominated world.

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South Africa

SIR—Britain took 96 years to get full suffrage, in four stages, the last only 58 years ago. During that time, poverty (far greater than that in South Africa today) virtually disappeared, owing to the gradual increase in wealth brought about by entrepreneurs. South Africa needs more trade and contacts with other countries so that it can continue to raise the average standard of living.

The advanced countries demand that South Africa make really major social changes much more quickly than they did; at the same time they apply sanctions that cripple its power to do so. Such behaviour may be explained, but not excused, by the campaign against South Africa in the media, especially television, during the past year or so. The letter of J.A. Barnes (*Nature* 324, 104; 1986) rather dispels the hope that an academic would show more understanding.

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AIDS in an African context

Clues to the origin of the virus that causes AIDS continue to emerge from studies of African viruses. But African countries have more pressing concerns.

SIR Fred Hoyle may hold the view that the AIDS (acquired immune deficiency syndrome) virus is of extraterrestrial origin, others may like to believe that it escaped from a germ-warfare laboratory (surely a better plot would involve an unscrupulous biotechnology company that made and released the virus but whose vaccine development department has been a failure), but in all likelihood the human AIDS virus is a descendant of a monkey virus. The link between the two may be the second type of AIDS virus, identified earlier this year in western Africa. That is one, but by no means the only, reason why there is an increasing interest in the study of AIDS in Africa.

The reason to believe that the second type of virus is a link between monkey viruses and HIV-1 (human immune deficiency virus type 1, otherwise acronymically termed HTLV-3, LAV or ARV) is that it is more closely related to the former than the latter, at least by relatively crude comparisons. But the situation is complicated by the isolation of two apparently different versions of the west African AIDS virus and of several apparently different monkey viruses.

On the evidence so far published, the two versions of the human virus are very similar in composition but only one causes AIDS. That one was isolated by Luc Montagnier's group from AIDS patients in Guinea Bissau and the Cape Verde Islands, and was named LAV-2 by them (Clavel, F. *et al. Science* **233**, 343–346; 1986). The group has since also isolated it from AIDS patients in neighbouring countries, renamed it HIV-2 and, as reported further on in this issue (Clavel, F. *et al. Nature* **324**, 691–695; 1986), molecularly cloned the virus. The other version of the virus has been found in nearby Senegal by Myron Essex's group and always in people without AIDS (Kanki, P.J. *et al. Science* **232**, 238–243; 1986). This virus is called HTLV-4.

Unfortunately, and largely because of the sorry state of affairs that exists between leading French and US AIDS researchers, there has been no direct comparison of HIV-2 and HTLV-4. Therefore it remains uncertain whether their obvious similarities betoken an identity — or rather a pseudoidentity, because, as Clavel *et al.* now report, different isolates of HIV-2 vary in much the same way as those of HIV-1. The more interesting possibility is that HTLV-4 differs from HIV-2 in

some crucial way that accounts for its apparent lack of pathogenicity.

Whatever the relationship of HTLV-4 and HIV-2, both seem more closely related to monkey viruses than they are to HIV-1. With their molecularly cloned HIV-2, Clavel *et al.* now confirm that relationship in terms of the degree of hybridization between the nucleic acids of HIV-2 and the virus that is the cause of the AIDS-like disease of captive rhesus macaque monkeys. This virus, usually known as STLV-3_{mac} but now called SIV (unwisely without a species designation) by Clavel *et al.*, is related both to a virus in healthy captive sooty mangabey monkeys and to a virus in healthy wild African green monkeys. It is with the latter, called STLV-3_{agm} and now molecularly cloned (Hirsch, V. *et al. Proc. natn. acad. Sci. U.S.A.* **83**, 9754–9758; 1986), that HTLV-4 has been compared and shown to be closely related. Hence the hypothesis that an apparently harmless monkey virus, STLV-3_{agm}, was the origin of an equally harmless human virus, HTLV-4, which evolved into the pathogenic HIV-2, the ancestor of HIV-1. Although this hypothesis is not without its problems, it also leads to some predictions that will be put to the test once all the viruses are cloned and sequenced. So far only HIV-1 is sequenced but the HIV-2 sequence is well on the way and the others should follow soon, if all goes well.

It is conceivable, at least, that this line of research will provide compelling evidence of an African origin of AIDS. Although that would not exactly be welcome news in Africa, at least the time is past when such a notion could lead to vehement denials, largely for fear of stigmatization and, it seems, loss of tourists. With a few exceptions, African countries are now frank about their AIDS problem, as well they might be given its scale.

Worst affected are central African countries, of which Kenya, Zaire and Uganda are the best studied. Reliable figures, which have been hard to come by, are summarized in two recent reviews (Quinn, T.C. *et al. Science* **234**, 955–963; 1986 and *AIDS and the Third World*; The Panos Institute*, 1986). As in developed nations, most of the data come from the major cities where the problem is at its greatest. The highest rates of infection are invariably in prostitutes, with figures ranging from 27 to 88 per cent. More

alarming are the figures of 10–20 per cent from blood donors and antenatal clinics, as these are not high-risk groups in Western terms.

All the African figures have to be taken in context. Is, for example, the high rate of infection in blood donors typical of the population from which they are drawn or is it exaggerated by infection in the course of previous blood donations? Are the high rates of infection in general the result of other sexually transmitted diseases which, because of the genital ulcerations they cause, facilitate infection by the AIDS virus? And what rate of increase underlies these figures? Unfortunately there are few longitudinal studies of infection in Africa yet, although Quinn *et al.* boldly state that the present annual incidence of infection is approximately 0.75 per cent among the general population of central and east Africa. Nor are there many random population surveys; but a 6 per cent rate of infection recorded in a neighbourhood survey in Kinshasa, Zaire, is an indication of how serious the problem is.

More serious still is the fact that even the most simple means of limiting the spread of infection are not routinely used in most central African countries. It is estimated that between 1,000 and 1,500 new infections a year would be prevented in one major hospital alone in Zaire if there was routine screening of blood donors for HIV-1. Only a few million dollars would pay for routine blood screening throughout central Africa. But the cost per test would be somewhere between 3 and 30 times the average sum spent on an individual's health care in Africa. The prospects of routine testing seem poor without the help of foreign aid. To put the overall financial problem even more starkly, according to Quinn *et al.*, "the cost of caring for ten AIDS patients in the United States (approximately \$450,000) is greater than the entire budget of a large hospital in Zaire, where up to 25 per cent of the pediatric and adult hospital admissions have HIV infection."

It is in this context that it is hardest to have much sympathy with some of the endless wranglings that go on concerning nomenclature of the viruses, with the neocolonial way in which some of the foreign interest in African AIDS is manifested, and with the lack of cooperation between rival groups when cooperation would further hasten understanding.

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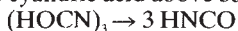
Fuel technology

Control of nitrogen oxides

A. Williams

AIR pollution arising from the emission of nitrogen oxides, NO_x , from combustion taking place in boilers, furnaces and engines, has increasingly been recognized as a problem (see, for example, *Fourth Report from the Environment Committee, Acid Rain*, HMSO, 1985). NO_x has been implicated as a photochemical oxidant and a constituent of acid rain, as well as being a pollutant in its own right. As a consequence, stringent legislation to limit its emission has been introduced or is proposed in the next few years in many parts of the world, but particularly in the United States, Japan and western Europe.

Several methods for controlling NO_x emissions have been developed (for example, *Control Technology for Nitrogen Oxide Emissions from Stationary Sources*, OECD, 1983). Most of these have some unsatisfactory aspects, however, and there is a constant search for new control technologies. Elsewhere in this issue R.A. Perry and D.L. Siebers (*Nature* **324**, 657; 1986) propose such a new technique. This method, developed at the Sandia National Laboratories, Livermore, California, is based on adding isocyanuric acid to hot exhaust gases, the acid being produced from the thermal decomposition of non-toxic cyanuric acid above 330 °C



Because this new control method has some attractive features it is worthwhile looking at it in the context of the control strategies that are currently available. The engineering aspects of the control strategies available for both NO_x and SO_2 are under investigation by the Fellowship of Engineering in the United Kingdom and its results will be published next year.

The major sources of NO_x are stationary combustion sources, such as boilers and furnaces, and motor vehicles, although some NO_x is emitted from chemical processing plants. The primary pollutant produced is NO , although some NO_2 and N_2O can be formed, and the NO reacts in the air to form a mixture of NO and NO_2 (NO_x). First, thermal NO is formed in combustion chambers from the oxidation of N_2 by the oxygen present in the 'excess air' needed to carry combustion to completion. Second, NO is formed from fuel-nitrogen compounds present in coal or to a smaller extent in oil, but not in natural gas. Third, NO can be formed in fuel-rich flames where hydrocarbon radicals react with nitrogen; this source, which is normally small, is termed 'prompt- NO '.

Various NO_x control technologies have been directed at combustion chamber modifications or a flue-gas treatment

called denitrification. The combustion chamber modifications involve techniques to reduce the flame temperature and/or to reduce the amount of oxygen available for primary combustion. In engines this can be achieved by lean-burn techniques or stratified combustion chambers. In boilers and furnaces it can be achieved by low NO_x burners that depend on carefully controlled mixing of fuel and air, by flue-gas recirculation, or by low-temperature combustion techniques such as fluidized bed combustion or catalytic combustion.

The NO_x regulations currently proposed are so strict that in the case of both engines and stationary combustion units it is becoming increasingly difficult to obtain the low concentrations of NO_x required by combustion modifications alone. Consequently, interest has centred on flue-gas denitrification for supplementing or replacing combustion control methods.

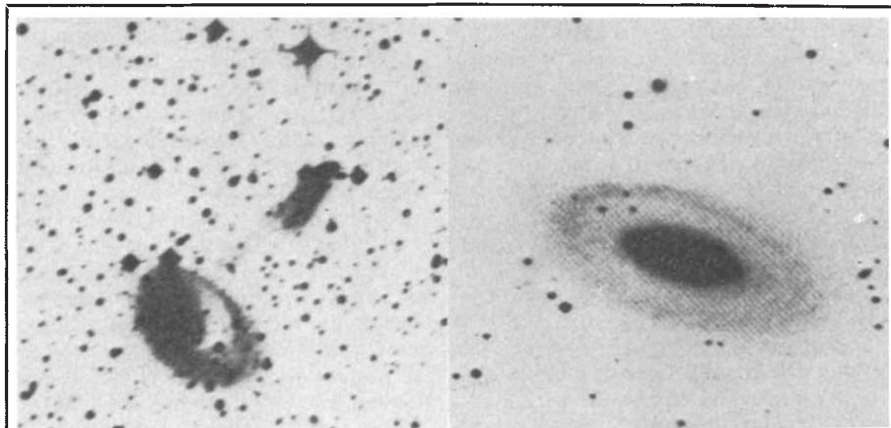
The methods available depend on the application and particularly whether the gases are clean or dust-laden. The most attractive methods involve: (1) heterogeneous catalytic destruction usually based on precious metal catalysts and involving the reaction $2 \text{NO} \rightarrow \text{N}_2 + \text{O}_2$; (2) ammonia injection, called thermal NO_x , based mainly on the reaction $\text{NH}_3 + \text{NO} \rightarrow \text{N}_2 + \text{H}_2\text{O}$ and so on; (3) a catalytic version of the ammonia process usually called selective catalytic reduction; and (4) injecting methane or similar hydrocarbons to 'reburn' NO by reactions such as $\text{CH}_4 + \text{NO} \rightarrow \text{HCN} + \text{CO} + \text{N}_2$. The first of these methods needs a cleanish gas,

such as engine exhaust or the flue gases from natural gas combustion, but needs controlled oxygen (air) addition. The other two need controlled ammonia or methane injection and controlled flue-gas temperatures and are really suitable only for large stationary plants. The applicability of many of these methods is limited where extensive heat recovery is required and the platinum-based catalysts, which operate down to 350 °C, look particularly attractive, if deactivation does not occur.

The new technique of Perry and Siebers therefore looks an attractive proposition. It provides a means of reducing NO_x levels down to those required by the most stringent regulations by passing the gas over the reactant without having to control the oxygen levels. It will operate over a temperature range of 450 to 900 °C, although at lower temperatures (100–450 °C) it actually produces NO_x itself. It seems clear that the work describes a technique that is a valuable additional NO_x control technology.

Their paper stems from earlier work (Perry, R.A. *J. chem. Phys.* **82**, 5485; 1985) that contains an interesting proposal about the chemistry leading to the formation of N_2O in some flames containing fuel-nitrogen. Although it has been known for several years that N_2O is formed during the combustion of organonitrates or nitrates (see, for example Gray, P. & Williams, A. *Nature* **188**, 56; 1960) it is a surprise that it can be formed in significant amounts as a result of the combustion of coal. The new work is therefore particularly interesting in that it proposes a control technology linked with these chemical routes and offers additional control mechanisms for future development. □

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JANE Few and Barry Madore, in a survey published in a recent issue of the *Monthly Notices of the Royal Astronomical Society* (222, 673–682; 1986), identify two sub-types of ring galaxies. On the left is a P-type ring galaxy showing the crisp, knotty structure of the ring itself, the displaced nucleus and companion galaxies. On the right is an O-type galaxy, showing the smooth structure of the ring and the centrally located nucleus. These data support the idea that P-type galaxies were formed from disk galaxies by the direct head-on collision with a companion and that O-type galaxies formed in isolation. □

Cellular signalling

A second messenger function for inositol tetrakisphosphate

Bob Michell

AN entirely novel concept entered our knowledge of how receptors send signals through membranes to the cell interior when it was realized that the receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate yields two second messengers rather than one. Inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) and 1,2-diacylglycerol have different molecular targets within the cell, but they often act synergistically during the evocation of cell responses (reviewed in refs 1–3). Enzymic removal of the 5-phosphate of $\text{Ins}(1,4,5)\text{P}_3$, discovered in 1982, provided a perfectly respectable mechanism for its inactivation. In the past year or so, however, it has become increasingly clear that there is a quantitatively important alternative pathway for the rapid metabolism of $\text{Ins}(1,4,5)\text{P}_3$ in stimulated cells, as a result of which it is phosphorylated to inositol 1,3,4,5-tetrakisphosphate ($\text{Ins}(1,3,4,5)\text{P}_4$), which is then dephosphorylated to inositol 1,3,4-trisphosphate, $\text{Ins}(1,3,4)\text{P}_3$ (see my recent News and Views article⁴ and refs 5,6). It has been widely expected that $\text{Ins}(1,3,4,5)\text{P}_4$ or $\text{Ins}(1,3,4)\text{P}_3$, or maybe both, would function as additional intracellular messenger(s) generated by this increasingly complex signalling system, Irvine and Moor⁵ now present the first evidence in support of this hope. Using sea urchin eggs, they obtain evidence that strongly suggests that $\text{Ins}(1,3,4,5)\text{P}_4$ regulates the entry of calcium ions into these cells from the external medium.

Whittaker and Irvine⁷ were the first to show that injection of $\text{Ins}(1,4,5)\text{P}_3$ into sea urchin eggs suspended in a Ca^{2+} -containing medium causes the raising of a fertilization membrane, a response which seems identical to that produced by normal fertilization by sperm. It is generally agreed that this response is mediated by a rise in intracellular Ca^{2+} that is fed both from internal Ca^{2+} stores and from the external medium. $\text{Ins}(1,3,4,5)\text{P}_4$ does not mobilize Ca^{2+} from intracellular stores⁶, so it was no surprise when it failed to activate eggs⁵. But it has been clearly demonstrated that $\text{Ins}(2,4,5)\text{P}_3$ mobilizes Ca^{2+} from the same intracellular store as $\text{Ins}(1,4,5)\text{P}_3$, so its failure to raise a fertilization membrane was unexpected⁵. Irvine and Moor surmise that the key to the difference between $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(2,4,5)\text{P}_3$ might lie in the ability of the former to be converted to $\text{Ins}(1,3,4,5)\text{P}_4$ in the eggs into which it is injected, and wondered whether activation requires the simultaneous presence within the eggs of

$\text{Ins}(1,4,5)\text{P}_3$ and of $\text{Ins}(1,3,4,5)\text{P}_4$ derived from it. ($\text{Ins}(2,4,5)\text{P}_3$ is not an effective alternative substrate for $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase, so it could not provide $\text{Ins}(2,3,4,5)\text{P}_4$ as an $\text{Ins}(1,3,4,5)\text{P}_4$ analogue.) To test this idea, eggs were incubated in a Ca^{2+} -containing medium and injected with a mixture of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(2,4,5)\text{P}_3$. The results are startling. This mixture of two ineffective inositol phosphates activates the eggs as effectively as $\text{Ins}(1,4,5)\text{P}_3$ alone. As with other modes of egg activation, success with the $\text{Ins}(1,3,4,5)\text{P}_4/\text{Ins}(2,4,5)\text{P}_3$ mixture requires the presence of extracellular Ca^{2+} . Irvine and Moor conclude⁵ that $\text{Ins}(1,3,4,5)\text{P}_4$ is likely to be the intracellular messenger responsible for initiating Ca^{2+} entry from the exterior, and that this is somehow dependent on the prior or simultaneous $\text{Ins}(1,4,5)\text{P}_3$ -triggered discharge of the intracellular Ca^{2+} store. They envisage that these two processes are coupled in some mandatory way such as

that envisaged by Putney⁸: his idea is that Ca^{2+} entering from the exterior does not move directly into the cytosol, but is routed through the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool, with influx particularly rapid when this pool is either partially or fully discharged.

If future data confirm this view of events in sea urchin eggs and extend it to other cells, we now have all the key elements necessary to understand receptor-regulated Ca^{2+} mobilization into the cytosol of most stimulated cells. Of course, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ need to be both produced and inactivated in a coordinated way. A remarkably tidy facet of this entire system is that the inositol polyphosphate 5-phosphatase that was originally detected as an $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase may simultaneously inactivate both of these signals. □

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Atomic motion

Quantum fluctuations in sub-micron wires

Patrick A. Lee

PHYSICISTS have long held the notion that measurements on a macroscopic sample are the same as the average over an ensemble of similarly prepared samples. In the past two or three years, this notion has been challenged by a combination of two factors: the ability to prepare small samples using micro-fabrication techniques; and the general availability of low temperature in the tens of millikelvin range. The typical sample dimension is 0.05 μm diameter and 1 μm long, so that these 'small' samples still contain 10^8 – 10^9 atoms. The surprise is that these samples, which are macroscopic on the atomic scale, already exhibit large fluctuations in their electric conductivity which also vary from sample to sample. Furthermore, theoretical developments have led to a quantitative description of this phenomenon, and point to the possibility of detecting the change in the conductivity of a small metallic sample if the sample is perturbed in the slightest manner, even in the limit of displacing a single atom.

These developments were initiated by the discovery that the resistances of small

metallic wires ($0.04 \times 0.04 \times 0.7 \mu\text{m}^3$) exhibit random structure when a magnetic field is applied perpendicular to the wires¹. The structure is observable below 1 K; it increases in magnitude down to 10 mK where it is about a 0.1 per cent effect, it is reproducible upon cycling of temperature and magnetic fields, and it is a signature of a particular sample.

At these low temperatures, the resistance is entirely due to the presence of impurities and imperfection in the sample and the motion of the electron through the sample must be described in terms of quantum mechanics. A particularly illuminating way of describing the low temperature conductance G (inverse of resistance) of a disordered sample was provided by Landauer², who showed that G is proportional to the transmission probability of the electron wave through the disordered medium. The proportionality constant is e^2/h which is a combination of fundamental constants with the dimension of conductance and equals $4 \times 10^{-5} \Omega^{-1}$. (The same combination is the unit of the Hall conductance in the quantized Hall effect.)

The first step towards understanding the experiment was taken by Stone³, who performed numerical calculations based on the Landauer formula for two-dimensional samples consisting of 40×400 sites and found fluctuations qualitatively similar to the experimental observation. The idea is that according to Feynman's formulation of quantum mechanics, the transmission probability is given by a sum of the quantum amplitudes for many

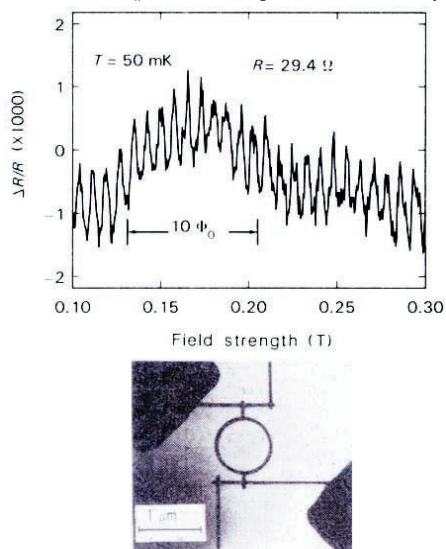


Fig. 1 Variation of the resistance (R) of a metallic ring (photographed in the inset) with magnetic field. The field strength is measured in tesla and is also shown in terms of the flux through the ring in units of the flux quantum $\Phi_0 = h/e$. The oscillation with period h/e is the Aharonov-Bohm effect and the random structure it rides on is the universal conductance fluctuation. The magnitude of both are of the order of $e^2/h \sim 4 \times 10^{-3} \Omega^{-1}$ in conductance units. The inside diameter of the ring is $0.784 \mu\text{m}$ and the width of the wires is $0.041 \mu\text{m}$. (From ref. 9.)

classical paths, and that the phase of each path is changed by the magnetic field in a random way. As a result the transmission probability and therefore G exhibit fluctuations as a function of magnetic field. However, numerical models showed a 10 per cent fluctuation and it was not clear how one could extrapolate results based on 10^4 sites to the experimental system with 10^6 atoms. One might have guessed that as the sample size increases, the fluctuation will average away, but the numerical work showed that the magnitude of the fluctuation is almost independent of sample size.

The answer to these questions was provided by Lee and Stone⁴ and independently by Altshuler⁵ who showed analytically that if the question is posed in terms of ΔG , the root-mean-square fluctuation in the conductance, instead of the percentage fluctuation, then at sufficiently low temperature ΔG is always of the order of e^2/h , independent of sample size and the amount of disorder, as long as disorder is weak and the sample is metallic. The re-

quirement of low temperature is that the electron should not suffer any inelastic collision as it traverses the sample. For a $1\text{-}\mu\text{m}$ structure, this requirement is usually satisfied below 10 or 100 mK. The prediction of this phenomenon of universal conductance fluctuation is consistent with both the numerical and experimental work and has now been verified in various experimental systems^{6,7}.

Universal conductance fluctuation is a consequence of quantum-mechanical coherence on a macroscopic length scale. A most dramatic manifestation of this coherence is the Aharonov-Bohm⁸ effect in normal metals, which was observed at about the same time as universal conductance fluctuation⁹. (The Aharonov-Bohm effect is the idea that the electromagnetic potential alone, in the absence of any electromagnetic field, causes an observable quantum effect — a shift in the phase of the wavefunction.) As shown in Fig. 1, a small metallic ring is fabricated and its conductance is measured as a function of magnetic field perpendicular to the ring. By extensions of Aharonov and Bohm's original arguments⁸ which applied to electron motion in free space, it was predicted theoretically¹⁰ that electron waves which propagate down the two arms of the ring would be phase-shifted by the magnetic field, and their interference would lead to periodic oscillation of the conductance. The observation of these periodic oscillations (Fig. 1) is a dramatic demonstration of quantum-mechanical coherence.

One might worry that the amplitude of the periodic oscillations may be greatly reduced due to cancellations between the many Feynman paths with random phase which propagate down the arms. But this is the same issue addressed by the theory of universal conductance fluctuation. Thus, the magnitude of the periodic oscillation should be the same as the random structure, that is, of the order of e^2/h . This is indeed the case for the data in Fig. 1.

The remarkable sensitivity of the conductance of a single sample to the magnetic field raises the following question: how sensitive is the conductance to a change in the atomic configuration of a sample? The question was answered by Altshuler and Spivak¹¹ and independently by Feng, myself and Stone¹² who showed that changing the location of a single atom in a sample can, under suitable circumstances, lead to a conductance change of e^2/h as well and that this change is independent of sample area for thin films.

This remarkable result is a natural consequence of the view of the electron motion as a superposition of many classical Feynman paths. The point is that the classical motion of an electron across a strongly disordered metal is a random walk, which explores a large portion of the sample. In fact, in two dimensions (thin films) the random walker visits a sizeable frac-

tion of all the impurities, so that disturbing a single impurity affects the phase of a sizeable fraction of all the Feynman paths. In other words, changing a single impurity has the same effect as replacing the entire sample. According to the theory of universal conductance fluctuation, this leads to a change of the conductance of the order of e^2/h for highly disordered metallic thin films and wires. Less dramatic, but still significant changes are predicted for bulk samples as well.

Ideally, one would like to design an experiment to test this prediction, but nature appears to be kind enough to have provided an example. Experimentalists at Purdue University¹³, studying the resistivity of small wires and thin films, were perplexed by their observation that at temperatures below 1 K their data were not reproducible from run to run. A more careful examination of the resistance as a function of time shows that it is time dependent. Figure 2 shows an example where the resistance makes two excursions in the period of an hour. In this example the resistance returns to the base line after a few minutes, but more complicated time dependence is also observed. The experimentalists interpreted these jumps as being caused by the motion of a single atom or a small group of atoms and the magnitude of the jump is consistent with the theoretical prediction. If this interpretation is correct, the motion of a single atom in a macroscopic metallic sample is indeed detectable. It is not known what caused the atomic migration in the bismuth films at these low temperatures, but the effect may allow study of the dynamics of such slow configuration changes.

Although the initial discovery of sample-specific fluctuations was made at low temperatures, it has become clear that quantum-mechanical coherence plays an important role as long as the inelastic scattering length greatly exceeds the mean free path, a condition which is satisfied

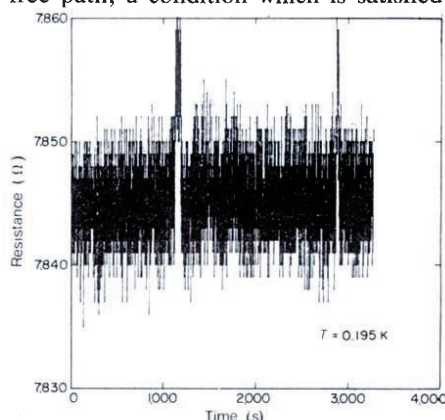


Fig. 2 The resistance of a bismuth film ($16 \times 8 \times 0.007 \mu\text{m}^3$) at 0.195 K as a function of time in seconds. The two excursions from the constant background at 1,100 and 2,900 s (rapid fluctuations are instrumental noise) are interpreted as being due to the motion of a single defect in the bismuth film. (From ref. 13.)

even at room temperature if the metal is sufficiently disordered. For example, Feng *et al.*¹² point out that the sensitivity to impurity configuration provides an estimate of the magnitude of $1/f$ noise due to defect motion in highly disordered metals. At the same time, it is likely that insights gained from the studies of electron wavefunctions will deepen our understanding of the propagation of classical waves such as electromagnetic or sound waves through a random medium. □

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Carcinogenesis

A superfamily of potentially oncogenic hormone receptors

Stephen Green and Pierre Chambon

UNDERSTANDING how patterns of gene transcription are controlled during the development of eukaryotic organisms and in terminally differentiated cells is an important goal of molecular biology. This control appears to be achieved through the interaction of *trans*-acting proteins with *cis*-acting DNA promoter elements, which may result in a positive or a negative effect (see ref. 1). Very few regulatory proteins have been characterized in higher eukaryotes. Steroid hormone receptors are such a family of regulatory proteins, whose ability to control gene expression is dependent on the binding of their specific ligand. Activation of transcription seems to result from the specific binding of the hormone-receptor complex to promoter 'enhancer' elements of target genes². Thus, the study of these receptors is of interest not only for elucidating how the receptor interacts specifically with its ligand, but also for understanding how this binding leads to the activation of transcription through specific protein-DNA interactions. New work from four laboratories reported in this issue³⁻⁶ brings us closer to understanding these mechanisms but also produces some apparent contradictions.

The recent cloning of glucocorticoid^{7,8}, oestrogen^{9,10} and progesterone^{11,12} receptors provides the first opportunity to investigate, at the molecular level, how transcription by RNA polymerase class B (II) is regulated in higher eukaryotes. Sequence comparisons¹⁰, together with mutation analysis^{13,14}, have defined two conserved regions that correspond to important functional domains (see figure). The putative DNA-binding domain is a highly conserved 66-amino-acid region (C) which has the potential to form two DNA-binding 'fingers' analogous to those proposed for the transcription factor

TFIIIA (see refs 15 and 16), but involving two pairs of cysteines instead of pairs of cysteines and histidines¹¹. Results obtained with a chimaeric receptor show that region C determines the specificity of the receptor for the transcription of target genes¹⁷. The hormone-binding domain (region E) is located in the C-terminal half of each of the receptors^{13,14}. The hydrophilic region D, which is variable in both length and sequence, may correspond to a hinge between the DNA- and hormone-binding domains. Whether region A/B is required for activating transcription is not clear because analysis using insertional mutants of the human glucocorticoid receptor¹⁴ indicates its importance for activation of transcription, whereas results obtained with deletion mutants of the rat glucocorticoid¹⁸ and human oestrogen (Kumar, V. *et al.*, in preparation) receptors suggest that it is dispensable.

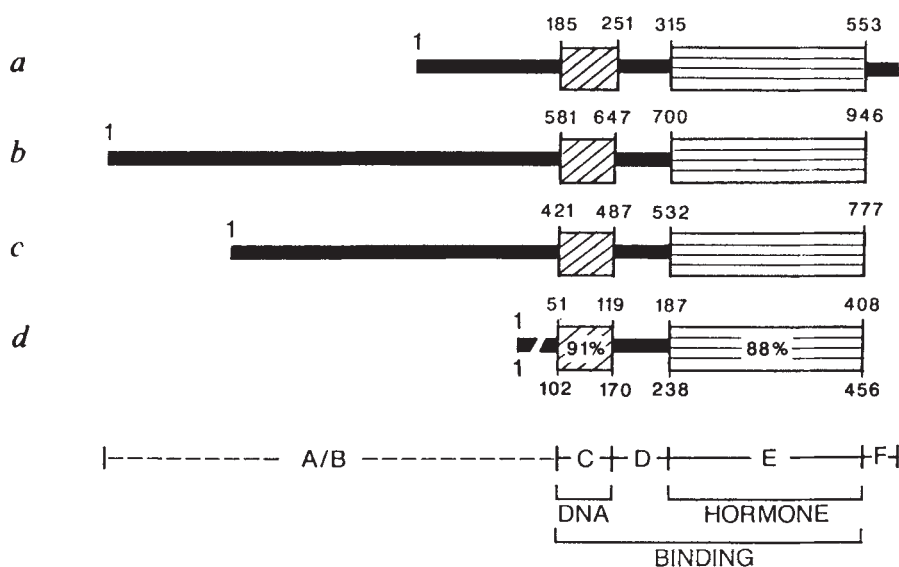
Sequence comparisons revealed similarities between the product of the *v-erb-A* gene of the avian erythroblastosis virus and the human glucocorticoid¹⁹ and oestrogen⁹ receptors (see figure). This similarity is most striking in region C which is equally conserved when comparing these receptors and *v-erb-A* (about 50–60 per cent in each case). Because significant homology is also found in region E (although to a lesser extent, see figure), it was suggested that *c-erb-A*, the cellular counterpart of *v-erb-A*, is a receptor for a steroid-related ligand¹⁰. Two reports on pages 635 and 641 of this issue^{3,4} demonstrate, surprisingly, that the product of both a chicken³ and a human⁴ *c-erb-A* gene bind specifically thyroid hormones with high affinity (although the affinity with which the two receptors bind T_3 differs by an order of magnitude), and may therefore be the chicken and the human receptors for those hormones. This

possibility is further supported by the nuclear localization of the chicken *c-erb-A* product.

The thyroid hormone receptor is known to be localized in the nucleus and it is believed that it modulates gene expression by a mechanism similar to that of steroid hormone receptors (see ref. 20). Regions C and E of the thyroid hormone receptor may thus correspond to their DNA- and hormone-binding domains, respectively. As region C of either the human and chicken oestrogen receptors¹⁰ or the human and rat glucocorticoid receptors²¹ are entirely conserved, it is surprising that C regions of the chicken *c-erb-A* and the human *c-erb-A* are only 91 per cent homologous, a value which is identical to the homology found between the progesterone and glucocorticoid receptors (see figure). These two *c-erb-A* products may thus correspond to different genes encoding receptors that bind the same hormones, but exhibit different target gene specificities. This possibility is supported by the finding of several *erb-A*-related genes located on difficult chromosomes in the human genome⁴. Studies on the transcriptional activation of thyroid hormone receptor-responsive genes using the two cloned *c-erb-A*s are required to demonstrate definitively that they correspond to functional thyroid hormone receptors. That steroid and thyroid hormones, which are neither structurally nor biosynthetically related, have receptors that have evolved from a common ancestor gene, suggests strongly that there is a large superfamily of genes whose products are transcriptional regulatory proteins. All these proteins probably have similar DNA-binding domains, but it will not be surprising to find that the activity of some of them may have become independent of ligand binding, thus allowing them to act as constitutive transcription factors. This superfamily could be comparable with that of the homeobox-containing proteins that are also involved in the control of gene expression during development, but whose putative DNA-binding domain may contain the prokaryotic helix-turn-helix DNA-binding motif rather than DNA-binding fingers¹⁶.

The classical model for steroid hormone action proposes that the hormone is required for translocation of the receptors to the nucleus and for its binding to the DNA-responsive elements of target genes (see ref. 2). This model is based on the observation that the hormone is required for the receptor to bind tightly to the nucleus. However, it has been shown more recently that the oestrogen (see ref. 22) and the progesterone (see ref. 23) receptors are localized, but weakly bound, in the nucleus even in the absence of hormone.

What, then, is the role of the hormone? Could the hormone-free receptor, for instance, occupy the DNA steroid-



Schematic alignment of steroid and thyroid hormone receptor sequences. The amino-acid sequences of the human oestrogen receptor (a), chicken progesterone receptor (b), human glucocorticoid receptor (c), and both the chicken (1-408) and human (1-456) *c-erb-A* proteins (d), were deduced from their complementary DNA sequences. Alignment is based on amino-acid similarity and the numbers refer to the position of amino-acid residues. The division of the steroid hormone receptors into 6 regions (A-F), based on a comparison between the chicken and human oestrogen receptors¹⁰, is shown at the bottom of the figure. The two highly conserved regions, representing the putative DNA-binding (region C) and hormone-binding (region E) domains, are shown as shaded blocks. Little or no significant homology is observed when comparing other regions of the receptors (solid black lines). Comparison between the chicken and human *c-erb-A* protein sequences shows 91 and 88 per cent sequence identity in regions C and E, respectively. Comparison between the various receptors in regions C and E show the following homologies (in per cent): C, steroid receptors/*c-erb-A* ~ 45-55; human oestrogen/chicken progesterone or human glucocorticoid receptors, 62; chicken progesterone/human glucocorticoid receptors, 91. Region E: steroid receptors/*c-erb-A* ~ 20; human oestrogen/chicken progesterone or human glucocorticoid receptors, ~30; chicken progesterone/human glucocorticoid receptors, 55.

responsive elements of target genes without activating their transcription? This important question is addressed in two papers on pages 686 and 688 of this issue^{5,6} and in another which has just appeared in the *EMBO Journal*²³. Using the *in vivo* dimethylsulphate footprinting technique, the group of Schütz⁷ shows that occupancy of the glucocorticoid-responsive element of the glucocorticoid-inducible liver tyrosine aminotransferase gene is influenced by the presence of the hormone and of its antagonist RU486. Protection of guanine residues within the glucocorticoid-responsive element is observed only in the presence of dexamethasone and not in the absence of hormone nor in the presence of RU486. Although a weak interaction of hormone-free glucocorticoid receptor with the responsive element cannot be excluded by these experiments, the results clearly support the notion that dexamethasone increases the affinity of the receptor for its target sequence.

In contrast, the two other reports present *in vitro* data which suggest that glucocorticoid⁶ and progesterone²³ receptors recognize preferentially their specific DNA-binding sites, irrespective of the presence of the hormone, and even when complexed with their common antagonist RU486. The group of Milgrom²⁴, which has studied the binding of highly purified

hormone-free rabbit progesterone receptor to specific fragments of the target uteroglobin gene, concludes that the affinity of the receptor for its target sequence is not modified by the presence of the hormone or of its antagonist, and that specific recognition is achieved even at 0°C. Willmann and Beato⁶ have approached the same problem by studying the ability of crude rat liver cytosolic preparations of hormone-free glucocorticoid receptor to bind to the responsive element of the mouse mammary tumour virus long terminal repeat sequence. Again, no effect of the hormone, or its antagonist, on the DNA-binding properties of the cytosolic receptor is found, although a heat-activation step (25 °C, 30 min) of the cytosol preparation is essential. Thus, it appears that the hormone is not necessary for specific DNA-binding *in vitro*, whereas it is *in vivo*.

There are at least two explanations that can reconcile these conflicting data. As proposed by both Willmann and Beato⁶ and Bailly *et al.*²³, the receptors may be complexed *in vivo* with some protein(s) or structure(s), preventing their binding to the target-gene responsive elements. Binding of the hormone to the receptor would induce the dissociation of such a complex, allowing the receptor to interact with its target sequences. One possible

candidate for such an inhibitor is the heat-shock protein of relative molecular mass 90,000 which, under certain conditions, readily associates with several steroid receptors²⁴. Alternatively, it cannot be excluded that extensive purification of the receptor or an *in vitro* heat-activation step may mimic the action of the hormone by artefactually inducing a conformational modification of the receptor required for recognition of specific target sequences. This modification could correspond either to the 'unmasking' of a previously hidden, but potentially active, DNA-binding domain, or to a conformational activation of a previously inactive domain. However, true affinity measurements (based on the 'on' and 'off' rates of the receptor to its target sequence) in the presence and absence of the hormone have not been performed in either of the two *in vitro* studies^{6,23}. This may explain some discrepancies between the results obtained *in vitro* by Willmann and Beato⁶ and those previously reported by Bourgeois *et al.*²⁵, who have also studied the effect of dexamethasone and RU486 on glucocorticoid-receptor binding to the mouse mammary tumour virus long terminal repeat. Thus, until real affinity measurements have been performed, it still remains an open question as to whether the efficiency of receptor binding to responsive elements is independent of the presence of the hormone *in vitro*. In fact the recent studies of Beato's group (personal communication) indicate that the *in vitro* 'on' and 'off' rates may be higher in the presence of the hormone. This may result in a marked effect on the occupancy of responsive elements by the receptors *in vivo* where the number of non-specific, compared with specific, DNA-binding sites is very large.

The avian *v-erb-A* oncogene, which cannot by itself induce cell transformation, enhances the erythroblast transforming potential of several primary oncogenes, such as *v-erb-B* (see refs 26 and 27). The *v-erb-A* gene product completely inhibits erythroblast differentiation into erythrocytes and allows transformed erythroblasts to grow in standard tissue-culture media, which has led to the notion³ that in erythroid cells, *v-erb-A* interferes with ion transport systems and regulatory factors of differentiation. Because of the sequence similarity between *v-erb-A* and steroid hormone receptors, it has been speculated^{10,19,28} that *v-erb-A* exerts its effect either by mimicking or by competitively antagonizing the effect of *c-erb-A* on the transcription of target genes. Assuming that the thyroid and steroid hormone receptors function similarly, it is intriguing that *v-erb-A* can exert its specific effects on erythroblasts even though it cannot bind thyroid hormones, presumably because of some of the many mutations present in its putative ligand-

binding domain (region E)³. It is likely that, because of other mutations²⁷, v-erb-A has acquired the ability to bind 'constitutively' to responsive elements of target genes. Whether this would result in v-erb-A mimicking or competitively inhibiting (for example, because the truncation of region A/B in v-erb-A would prevent it from activating transcription) the effect of c-erb-A, and/or whether the two amino-acid substitutions that are present in the putative DNA-binding domain of v-erb-A³ have resulted in changing its target gene specificity when compared with c-erb-A, remains to be seen.

If one assumes that the thyroid hormone receptor acts like steroid hormone receptors, the reports of Weinberger *et al.*⁴ and Sap *et al.*³ in this issue suggest that 'altered' enhancer factors may be important in oncogenic transformation by interfering with the transcriptional regulation of crucial target genes. The well-known effect of the presence of oestrogen receptor on the growth of some human breast cancers (see ref. 29) and the insertion of the hepatitis B viral genome into an erb-A-related gene of a human hepatoma³⁰ suggests that the superfamily of receptor genes contains several potential oncogenes encoding enhancer binding factors. The polyoma virus enhancer can be activated *in trans* by expression of the c-Ha-ras oncogene and by the tumour promoter TPA³¹. Furthermore, the activity of several viral and cellular enhancers can be repressed *in trans* by expression of the adenovirus immortalizing oncogene E1A (see ref. 32). It may be possible, therefore, that alteration of enhancer elements of key cellular genes and/or of their binding factors correspond to crucial steps in

many types of carcinogenesis.

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Cosmology

Views with a gravitational lens

Rachel Webster and Michael Fitchett

GRAVITATIONAL lensing should in principle provide a fundamentally new technique for exploring the distribution of mass in the Universe, for determining cosmological parameters and for determining the properties of background sources. For example, model fitting can determine the mass distributions of specific lenses and statistical studies can be used to determine the existence and density of populations of compact objects, strings or clumped dark matter. In theory, constraints can be placed on the cosmological parameters H_0 , Ω and Λ , but the realization of such measures may be difficult. The amplification bias introduced by gravitational lensing will affect source counts of background objects and may either be skewing what we observe, or may serve as a natural telescope to probe the structure of objects

at high redshift.

The central point that emerged from a recent workshop*, however, was the need for verification that supposed gravitational lenses are indeed what they have been claimed to be. Because one can in principle learn a great deal from either a particular instance of gravitational lensing, or a well-defined sample of multiply imaged quasars, it is important that a strict procedure is developed to confirm the status of a lens. When the first candidate lens, 0957+561A,B, was discovered¹ in 1979, it was subjected to rigorous scrutiny. Observations in a range of wavelengths and the identification of a reasonable candidate for the lens object have served to streng-

then the case that the two images observed are of the same quasar. Of course, if one hopes to learn something about the distribution of dark matter, the identification of a candidate lens object should not be a requirement, and the emphasis switched to other properties of the system. A further eight candidates have now been added to the list. It was not until the wide separation lens 1146 + 111B,C (at an image separation of 157 arc s it is about 20 times larger than any of the previous candidates) was mooted² that rigorous testing was again considered.

Both necessary and sufficient conditions for lens identification were discussed by Jacqueline Hewitt (Haystack Observatory) and Irwin Shapiro (Harvard). Multiple images are seen along different photon paths. This gives rise to spectral differences between the images, a time delay and perhaps a velocity difference. If there is intrinsic variation in the source, not only will the spectra be different at one instant in time, but each will also vary with time. Variation in the lens potential will change the relative amplifications of the images with time, and in particular amplifications of different regions of the image. Once the tolerances in velocity, spectra and spectral changes for two images of the same quasar have been calculated, a sufficient set of conditions for the positive identification of a lens needs to be determined. Possible tests include redshift, spectra, image morphology, identification of the lensing object and its effect on other background sources, time delay, polarization and parity. The last two tests can easily be applied only to radio quasars. Where such data are available, as in the case of 0957+561A,B, the case for lensing becomes very strong. For optical quasars however, if no deflecting object is seen, definitive identification relies not only on determination of the redshift and spectra, but also on the measurement of a time delay. As there are ten known optical quasars to every one known radio quasar, this ratio will be reflected in gravitational lenses, and an efficient technique for verifying optical candidates is required.

Reports of systematic surveys for multiple images by four groups were highlights of the meeting. Hewitt reported on a survey³ with the Very Large Array. This collaboration has identified a good candidate lens, 2016 + 112, and Hewitt described a further two candidates. This survey is a sensitive probe of matter clumped on scales of 10^{10} – 10^{12} times the mass of the Sun, and already the authors can derive a conservative upper limit of $\Omega_{\text{lens}} \leq 0.7$ for masses in this range. Marc Gorenstein (Harvard) described a new survey using archive Very Long Baseline Interferometry data which will be sensitive to multiple images in the range 0.1–1 arc s. This is the range of separations expected by galaxies acting as lenses. John Stocke (Joint Inst.

*The gravitational lensing workshop was held at the Department of Astronomy, University of Toronto and the Canadian Institute for Theoretical Astrophysics on 3–4 October 1986.

of Lab. Astrophysics) reported a study of X-ray active galactic nuclei selected near bright foreground galaxies. These sources show some evidence, both in redshift distribution and in the ratios of X-ray to optical fluxes, of being micro-lensed by stars in the foreground galaxy. One of us (R.W.) described an automated survey for optical candidates for gravitational lensing. This study uses the automated plate measuring machine at Cambridge, United Kingdom automatically to select quasar candidates with possible image multiplicity. Already this study can place limits on the numbers of lenses with separations in the range 10 arc s to 2 arc min (there are no candidates in a sample of about 2,500 quasars), and it can accumulate a substantial statistical sample of gravitational lenses in time.

Apart from the surveys, it became apparent that two other systematic monitoring projects are required. First, the known candidates need to be regularly monitored to determine time delays. C. Vanderreist (Meudon Observatory) presented data for 0957+561 collected over a six-year time base. So far a unique time delay cannot be determined from the data.

Second, as Shapiro emphasized, none of the data collected so far gives a definitive measure of the time delay; to make use of information about the individual variations more needs to be known about the general population of quasars. Not only do we need to know how quasars vary spectrally one from another, but also we need to know more about the power spectrum of the intrinsic variability.

Peter Schneider (Joint Inst. of Lab. Astrophysics) distinguished between linear and non-linear cases of lensing. In the former case, the optical depth in lenses is small, and reasonable progress can be made in analytically solving problems such as the effect of gravitational lensing on source counts. In the non-linear case more than one strong lens event affects the photon path, and global problems need to be solved numerically. A particular example of the latter is micro-lensing where the photon beam passes through a dense field of compact objects. A second area of theoretical work is the modelling of specific instances of multiple images. In most cases the modelling proceeds by trial and error until a single solution is found. Such solutions are never unique, and in fact no systematic method for examining the ensemble of possible solutions has been developed. Roger Blandford (California Institute of Technology) and colleagues are evaluating image parameters for different lens potentials to allow the most probable mass distribution in the lensing object to be determined.

Third, the effects of a clumped matter distribution on the measurements of cosmological parameters was discussed by Charles Dyer (Toronto). In particular,

many weak lenses can significantly affect background source amplifications¹. Myeong-Gu Park (Princeton) described a calculation which uses the known separations of double images to place limits on the cosmological parameters Λ and q_0 ; in particular $q_0 \geq -2.35$.

Four new applications of lensing were discussed. Bohdan Paczyński (Princeton) discussed the possibility that some of the gamma-ray bursts observed may be multiple images of the same event. Such an observation would be evidence for clustered micro-lenses; the main difficulty in verifying this hypothesis is the determination of an accurate position for each individual burst. Robert Wagoner (Stanford) explored the possibility of using supernovae as probes of dark compact objects. He presented detailed calculations of the light curves expected for Types I and II supernovae which had been micro-lensed, and discussed the possibility of actually observing such an event in a distant galaxy. Ulf Borgeest (Hamburg) described a parallax method, requiring a baseline of about 0.1 AU, which would allow the determination of both the size of the quasar emitting region as well as the trans-

verse velocity of the lens. Borgeest also reported a method for using time delays to determine the mass of the primary galaxy in the lens.

The wide separations of the observed doubles, and the absence in most cases of a suitable candidate for the lensing object, pose significant problems. Blandford pointed out that if quasars are clustered like galaxies then one might predict a few correlated quasar pairs in the known sample of quasars⁵. If, however, the multiple images without suitable lens objects are gravitationally lensed, then the mass distributions of the lenses have high mass-to-light ratios and cannot be composed of normal stellar material. Thus the verification of candidate lenses becomes of central importance. □

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Developmental biology

New data from mammalian homoeobox-containing genes

Michael H.L. Snow

SERENDIPITY gave us the homoeobox¹, a highly conserved DNA sequence of about 180 base pairs, first found in several *Drosophila* genes that control segmentation. Homoeoboxes were also detected in other segmented invertebrates and in several vertebrates including mammals, giving rise to the view² that this small DNA segment would serve to identify segmentation genes. This notion has since been revised because echinoderms and molluscs, not generally regarded as segmented, also have homoeobox-containing genes³. The recent demonstration⁴ that the *Drosophila* homoeobox-containing gene

Zerknullt (*zen*) is transcribed only in dorsal regions without segment specificity suggests that these genes have a wider scope than the organization of the anterior-posterior axis of the embryo. The new data of Gaunt *et al.* and Mavilo *et al.* on pages 662 and 664 of this issue^{5,6} add to our understanding of the specificity of expression of mouse⁵ and human⁶ homoeobox-containing genes.

The possibility that homoeobox-containing genes specify positional information in the embryo is still high and it is this aspect of patterning in morphogenesis that underlies the considerable interest in

Mouse homoeobox-containing genes

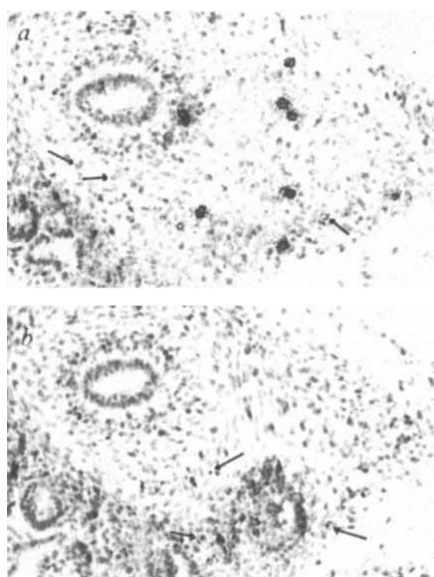
Name of gene	Chromosome	Synonym	Ref.	Best homologies (per cent nucleotides : amino acids)		
				<i>Drosophila</i>	Human	<i>Xenopus</i>
<i>Hox-1</i>	6	<i>m6</i>	13	Antp(82:95)	Hu 2(80:88)	MM3(79:83)
		<i>m5</i>	14	Antp(81:95)	Hu 2(83:90)	MM3(82:93)
		<i>m2</i>	14	Antp(??:??)	?	?
		<i>Hox-1.3</i>	15	Antp(73:82)	Hu 1(82:90)	MM3(74:82)
		<i>Hox-1.4 (mo10)</i>	2,5	Antp(70:74)	?	?
<i>Hox-2</i>	11	<i>Hox-2.1 (mul, H24.1)</i>	16,17,18	Antp(87:90)	Hu 1(92:95)	MM1(??:87)
		<i>Hox-2.2</i>	16	Antp(80:??)	Hu 2(91:100)	?
		<i>Hox-2.3</i>	16	Antp(77:??)	?	?
		<i>Hox-2.4</i>	16	Antp(??:??)	?	?
		<i>Hox-3 (m31)</i>	9	Antp(73:80)	?	?
<i>Hox-3</i>	16					
<i>En-1</i>	10	<i>Mo-en.1</i>	7	<i>en</i> (65:73)	?	?
<i>En-2</i>	5?	<i>Mo-en.2</i>	7	<i>en</i> (??:??)	?	?

mammalian homoeoboxes. It has not been possible so far to analyse developmental mutants in mammals (principally in the mouse) with the thoroughness that has been applied to *Drosophila* but it is hoped that the homoeobox will enable us to identify and isolate such genes. So, how far have we got?

Six homoeobox-containing genes have been identified in the human and a dozen in the mouse (see table). As in *Drosophila*, these genes seem to be clustered and to show temporal and spatial restriction in transcription. *Hox-2.1* transcription, for example, is detectable in mouse embryos as early as 7.5 days post coitum (during late primitive streak stages of gastrulation), shows peak levels in 11.5–12.5-day-old embryos and is enriched in spinal cord. Recent work with *Hox-2.1* using Northern blot analysis and *in situ* hybridization detects transcripts in embryonic kidney and in the mesodermal component of the developing lung (P. Holland, B. Hogan and R. Krumlauf, in preparation) and show also that transcription is temporally related to the differentiation of F9 embryonal carcinoma (EC) cells (R. Krumlauf, personal communication). In this latter system the duration of the transcription phase and its position in the differentiation sequence is related to whether differentiation is towards visceral or towards parietal endoderm. The mouse *Mo-en.1* gene is also differentially expressed during differentiation of EC cells⁷. Gaunt *et al.*⁵ in this issue describe similar differential expression of the mouse *Hox-1.4* (*Mo10*) gene by *in situ* hybridization. Here, transcription is again detectable at 7.5 days and is later found confined to spinal cord, mesonephric kidney and somites. The human homoeobox genes described by Mavilio *et al.*⁶ in this issue likewise show considerable stage and organ specificity in expression. All mammalian homoeobox genes show similar restrictions in expression to some extent. What do the data tell us?

The short answer is very little beyond what I have discussed above. One problem is the difficulty in defining comparable aspects of mammalian and *Drosophila* development, and this is severely complicated by technical limitations in the analysis of the mammalian embryo. The extremely powerful genetic analyses in *Drosophila* support and are reinforced by the ability to detect the earliest transcriptional activity by Northern blot analysis, to locate it accurately by *in situ* hybridization, and to locate translation by detection of protein products. The data are consistent, showing the onset of gene activity — with known developmental consequences — at or shortly after cellularization of the blastoderm.

The luxury of a four-pronged attack is not possible in the mammalian embryo. No genes are clearly definable as homoeo-



Kidney of 14.5-day-old mouse embryo. *a*, *In situ* hybridization with the anti-sense probe to mouse ϵ -globin. Heavy label localized over nucleated erythrocytes of yolk-sac origin. Arrows indicate three enucleated erythrocytes that originated from the liver. *b*, Control hybridization on adjacent section with the sense strand. Arrows indicate three nucleated yolk sac-derived erythrocytes which show up as positive in *a*. Probe donated by Andy McMahon, hybridization by Peter Koopman and Alex Reid.

tic or segmentation, although there are candidates for the latter⁸, and no antisera against homoeobox gene products have yet been described. So we have only the relatively insensitive transcription analyses (Northern blots and RNase protection assays) and the potentially very useful *in situ* hybridization techniques. At the moment neither technique alone tells us very much. Transcription analysis requires that tissues can be dissected in sufficient quantity and is therefore applicable only to late embryonic or fetal stages if spatial distribution of transcripts is to be studied. This is long past the time that positional cues associated with lineage definition are likely to be needed. Furthermore, any small population of positive cells, for example, stem cells, may well be swamped by a mass of non-transcribing tissue. For earlier, more interesting stages, *in situ* hybridization is the technique of choice: the 7.5-day-old mouse embryo will yield less than 20 ng of messenger RNA and although it is possible to accumulate material for transcription analysis from parts of these embryos it is clearly a daunting task and not suited to the present impatient scramble to publish homoeobox data. The *in situ* hybridization technique is difficult to apply to mammalian embryonic tissue. Non-specific binding is a common, but recognizable, artefact⁹ so it is imperative that rigorous control hybridizations, which should include a probe whose distribution is predictable in addition to the non-hybrid-

izing, non-complementary 'sense' strand of the probe under study, are not only carried out but also displayed alongside the hybridization with the complementary anti-sense strand of interest.

It is possible, when cell discrimination is helped by histological parameters, to locate accurately *in situ* hybridization signals to individual cells (see figure), but where morphology does not assist it can be far from easy. Even if such data were to be acquired for 7.5-day-old mouse embryos it would then leave the not inconsiderable problem of relating any patterns found to known organ-specific transcription shown in fetal stages. The fate maps and lineage data that exist for these early stages only relate to development up to about the 10-somite stage¹⁰; that is at least 24 hours before the primordia of many organs currently associated with homoeobox transcripts are found and 3–4 days earlier than the regional transcription analyses. Meanwhile, there are groups of apparently disparate organs expressing homoeobox genes at various developmental stages and we have little idea of what, if any, common feature they reflect. There are representatives of induced and inducing tissues, derivatives of different primary germ layers and organs which seem temporally and spatially unrelated in their origins. It may be a misleading coincidence, but the homoeobox-positive tissues equate roughly with those organs with determinate numbers of structural units, and negative tissues seem to be those that retain hyperplastic abilities throughout life¹¹.

Drosophila shows determinate development from the blastoderm stage. Although the information from *Drosophila* is not entirely straightforward¹², with curious gene interactions requiring explanation, the general picture is clear. The distribution of homoeobox gene transcripts is adequately linked through fate maps and genetics to later phenotype. There is some way to go before a similar state is reached with mammals and before we understand what their homoeobox-containing genes may do. □

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Mitochondrial DNA

Rearranging the plant genome

Ronald A. Butow

THE tendency of many plant mitochondrial genomes to undergo extensive rearrangements provides a satisfying framework for understanding the phenomenon of cytoplasmic male sterility (CMS), a maternally inherited defect common to many higher plants that impairs the development of pollen. Some extraordinary examples of the molecular basis for CMS in plants such as maize, sorghum and petunia were provided at a recent conference*.

The mitochondrial genomes of higher plants tend to be very large (200–2,400 kilobases) compared with other organisms. The presence of many direct repeats within some plant mitochondrial DNAs can lead to a profusion of subgenomic units that arise by recombinations across the repeat elements, as was shown for maize (D. Lonsdale, Plant Breeding Institute, Cambridge), wheat (F. Quetier, Université de Paris-Sud; L. Bonen, University of Ottawa) and *Oenothera* (A. Brennicke, Universität Tübingen). A somewhat simpler picture obtains for species of *Brassica*: the mitochondrial genomes of related species are roughly the same size, but they vary in the number and distance between repeats on the 'master' mitochondrial genome. This variation neatly accounts for the number, size and, in some cases, complete absence of subgenomic elements (J. Palmer, University of Michigan).

As if the presence of repeats leading to recombinational partitioning of plant mitochondrial genomes is not complex enough, on closer scrutiny, other substoichiometric sets of differently rearranged species are also apparent in maize mitochondria (P. Issac, University of Edinburgh). These substoichiometric populations, called sublimons, are maintained through many generations, and thus must somehow compete with the 'major' mitochondrial DNAs in that plant. Conventional thinking about the drive to segregate pure populations of mitochondrial genomes must be refined to consider the origin of sublimons by continued recombination or replicative persistence, and perhaps occasional amplification, in which they may eventually take over the population.

So where does CMS fit into this picture of a promiscuously rearranging genome? In maize, CMS is associated with C, T and S cytotypes, whereas normal male-fertile strains are designated N. Compared with N strains, mitochondrial DNA from C male-sterile cytoplasm shows three striking rearrangements that result in the for-

mation of chimaeric genes (R. Dewey, North Carolina State University): first, the 5' flanking region of the ATPase 9 gene has been replaced by sequences of unknown origin; second, the 5' ATPase 9 sequences plus a small amount of the coding region are now found upstream of an open reading frame (ORF) originating from the chloroplast genome, which, in turn, is fused in-frame to the ATPase 6 gene; and third, the replaced ATPase 6 sequences are fused in-frame to the cytochrome oxidase 6 gene, whereas the displaced cytochrome oxidase 6 sequences end up in a region with no ORF.

The mitochondrial genome from T strains, which are not only male sterile, but are also susceptible to toxin from the fungal pathogen *Helminthosporium maydis*, contains a rearrangement that is perhaps more bizarre than C. No less than seven recombination events must have taken place to create a chimaeric gene containing ATPase 6 sequences, a region 3' to 26S ribosomal DNA, a portion of the interior of 26S ribosomal DNA and a transfer RNA (Arg) derived from the chloroplast genome. Earlier work (C. Leaver, University of Edinburgh) showed that a novel polypeptide of relative molecular mass 13,000 was synthesized in the mitochondria of T strains. It now seems clear that this polypeptide is the product of this chimaeric gene, also called ORF 13. A precise correlation can now be made between restoration of fertility and expression of ORF 13. In one case of a fertile, toxin-resistant mutant of T, two changes were detected in ORF 13: a single G-to-A transition in the reading frame; and an adjacent 5-base pair insertion creating a frameshift, which may have occurred by a gene conversion from flanking DNA sequences 3' to the 26S ribosomal RNA gene (R.P. Wise, University of Florida). In other cases, mutation to fertility is caused by additional rearrangements that result in the deletion of the novel gene (W. Rottman, Plant Breeding Institute, Cambridge; C. Fauron, University of Utah). Restoration of fertility can also come from post-transcriptional processing activities that reduce the levels of translatable ORF 13 messenger RNA (Dewey; A. Abbott, Clemson University).

The picture is less clear for CMS-S. Male steriles of the S type have two linear DNA plasmids in their mitochondria, S1 and S2, that can integrate into the mitochondrial genome. In some nuclear backgrounds, reversion to fertility is always associated with the loss of S1 and S2 (E. Earle, Cornell University), although

this is not always the case in other nuclear backgrounds (S. Gabay-Laughnan, University of Illinois). S-specific gene products have been identified (G. Zabala, University of Illinois; Leaver), but where S1 and S2 are present in fertile revertants, there are no apparent differences in the synthesis of the plasmid-encoded proteins examined so far.

That the nuclear context can be important in expression of the CMS trait is also evident from studies with sorghum. A variant cytochrome oxidase subunit I gene has been identified (J. Bailey-Serres, University of California, Berkeley) containing both 5' and 3' rearrangements. These result in the synthesis of a long form of the protein (relative molecular mass 70,300) — the largest cytochrome oxidase subunit I polypeptide identified to date. Although the precise relationship of this rearrangement to CMS is not completely known, some lines containing the variant gene are male sterile whereas others are fertile. In either case, there are no obvious effects on cytochrome oxidase activity.

Petunia provides another example of the association of CMS with mitochondrial gene rearrangements (S. Izhar, Volcani Center, Israel; M. Hansen, J. Rasmussen and H. Nivison, Cornell). Here, recombination leads to the formation of a chimaeric gene between ATPase 9, cytochrome oxidase subunit II (COII) and DNA sequences from an unidentified reading frame. This fusion gene is expressed in male-sterile lines, as are the intact copies of ATPase 9 and COII genes. Expression of the chimaera seems to be unaffected in the presence of a dominant nuclear restorer gene, but preliminary evidence suggests normal COII expression is increased in a restorer background (Nivison).

It is clear from most of the evidence presented that some restorer genes function either by lowering or eliminating the expression of novel proteins encoded by rearranged mitochondrial DNA in male-sterile lines or, in some way, by overriding their presence. It seems reasonable to imagine that these novel proteins, which are probably located in the inner mitochondrial membrane, cause some mitochondrial dysfunction that becomes limiting at a critical step in microsporogenesis. But what this dysfunction might be is a complete mystery. In a related maternally inherited phenomenon in maize called non-chromosomal stripe, novel mitochondrial DNA rearrangements were identified — although not in any known genes — that result in severe alterations in mitochondrial (and chloroplast) morphology (K. Newton, University of Missouri). Because these plants show abnormal growth, a more immediate effect of mitochondrial dysfunction seems likely. □

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*International Workshop on Higher Plant Mitochondrial DNA 19–24 October 1986, Airlie, Virginia, USA.

Preservation in bogs

SIR—Doran *et al.* (*Nature* **323**, 803; 1986) have shown reasonable histological preservation of 8,000-year-old brain tissue from a bog site, yet they have not given an explanation for why the tissue preservation should be so good. We suggest that it is due to tannic acid and related compounds contained in the bog fluids. We have demonstrated excellent ultrastructural preservation of osteocytes from the 11,000-year-old antlers of an extinct Giant Irish Deer. In this case preservation in the bog environment is so good that cytological features, such as mitochondria, rough endoplasmic reticulum, Golgi elements and nuclear pores are clearly demonstrated.

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How many reactor accidents?

SIR—Edwards¹ is correct that Islam and Lindgren² have failed to recognize that their distribution function is a likelihood function. But he is incorrect in asserting that their conclusions are unwarranted and in assuming that physicists do not know how to work with small samples. In fact Islam and Lindgren's likelihood function has been used in particle physics for over fifty years to make estimates of particle decay rates when the number of events in an experiment is small³. Their approach yields not only estimates of the accident rate and probabilities but also the familiar standard errors and confidence limits without resort to Edwards' less familiar support function.

The estimate of r , the accident rate, is given by the maximum of the likelihood function. The standard error is conventionally quoted as the value(s) of r for which the likelihood L , falls to $e^{-1/2}$ of its maximum value. And the (one-tailed) confidence limits on r are set by solving for r in the integral equation:

$$\int_0^{\infty} L_N dr = P$$

where L_N is the normalized likelihood function. These values of r can then be used to obtain estimates, standard errors and confidence limits on the probability P_T of accidents within the next T years.

The two events over a cumulative 4,279 reactor years of operation⁴ give the estimate $r=1/2,140^{+2,600}_{-1,000}$ reactor years with a one tailed 95% confidence limit $r>1/5,240$ reactor years. In the next ten years 28

reactors are expected to be decommissioned and 156 are expected to come on line giving 4,900 reactor years of operation⁴. The probability of one or more accidents in the next ten years is then $P_{10}=0.90^{+0.09}_{-0.26}$ with a 95% confidence level of $P_{10}>0.60$. Thus one has a quantitative estimate of how small the probability of accident is likely to be.

Given that there have been two major accidents in the world's reactor inventory over 4,280 reactor years of operation the odds are 20:1 ($p>95\%$) that in 100 identical ensembles of reactors 60 ensembles will have one or more accidents within ten years. A similar calculation shows that even if the world's reactors are phased out over the next 25 years the probability of major accident is $P_{25}=0.994^{+0.005}_{-0.09}$ with $P_{25}>0.88$ at 95% confidence.

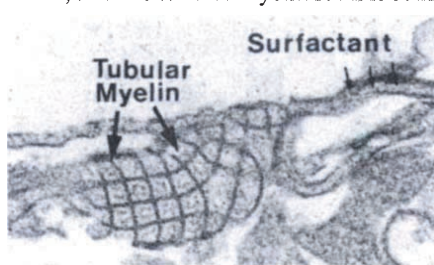
JOSEPH SCHWARTZ

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Alternative solutions

SIR—The small grid-like mystery object of Wolstenholme *et al.*¹ may be a fragment of 'tubular myelin', which is the contracted or non-spread form of lipoproteinaceous pulmonary surfactant. Fetal surfactant has been identified in human amniotic fluid², and the tubular myelin form is com-



Transmission electron micrograph of tubular myelin in the lung of a rat exposed to asbestos. Taken from ref.3.

monly observed. The dark-stained squares probably represent protein which is perhaps stained during chromosome preparation.

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1. Wolstenholme, J. *et al.* *Nature* **323**, 300 (1986).
2. Hook, G.E. *et al.* *Am. Rev. resp. Dis.* **117**, 541–550 (1978).
3. Brody, A.R. *et al.* *Fedn. Proc.* **44**, 2596–2601 (1985).

SIR—Wolstenholme *et al.*¹ have probably observed a well-preserved area of a meshwork of the nuclear lamina lining the nucleoplasmic surface of the inner nuclear membrane which supports the interphase chromatin. It is unusual to observe any remnant of lamina in a metaphase prepa-

ration, but two chromosomes in the figure appear to be sticking to lamina.

Full boxes in the pattern could represent the cytogenetic manifestation equivalent to the points of anchorage of the interphase chromosomes (telomeric ends and fragile sites) onto the laminar surface, thus reflecting the packing of chromosomes during interphase in a 'coded' form. The average crossover space of the human lamina is 0.52 μ m. A 'decoded' picture of the pattern of native nuclear lamina of *Xenopus laevis* oocytes can be found in a past issue of this journal².

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1. Wolstenholme, J. *et al.* *Nature* **323**, 300 (1986).
2. Aebi, U. *et al.* *Nature* **323**, 560 (1986).

Archaeopteryx, the primordial bird?

SIR—In view of the highly critical review of our book *Archaeopteryx, The Primordial Bird: A Case of Fossil Forgery* (*Nature* **324**, 185; 1986) I would like to bring to your notice recent developments that would seem to have vindicated our views.

The issue hinges on whether or not the material on which feather lines are impressed, and of which a few extraneous blobs exist elsewhere on the fossil surface, is comprised of a cement which is foreign to the rock. At our request the museum authorities supplied us with a sample of the suspect material and of the native rock matrix. These samples have now been examined by Dr Lee M. Spetner and his colleagues in Israel using a scanning electron microscope and X-ray spectroscopy. The sample from the rock matrix shows a characteristic crystalline structure exactly as in other specimens of Solnhofen limestone, with identical X-ray resonance spectra. But the sample from the suspect material shows a non-crystalline structure resembling that of amorphous material bound by an organic glue. X-ray resonance spectra showed large amounts of silicon as well as lead and chlorine which are certainly alien to native Solnhofen limestone.

These striking differences in texture and composition between the suspect regions and the native matrix are, in our view, a strong indication that this dispute will eventually be resolved in our favour. The results have been communicated to the British Museum along with a request for further samples of material from the fossil for examination with a view to narrowing down the possible organic compounds that are involved in the 'glue'.

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Who shall be the judge?

Frederick Dainton

The Science Critic: A Critical Analysis of the Popular Presentation of Science.
By Maurice Goldsmith. Routledge & Kegan Paul: 1986. Pp. 217. £15.95, \$35.

It is a truism, forever fixed in our childhood minds by the story of Pandora's box, that knowledge can be used for good or ill and that the problem for human societies is to maximize the former and minimize the latter. In the eighteenth century this problem was sufficiently evident to Dr Johnson, when he astounded the world by completing his book *Rasselas* within a week, to invent a philosopher who would not communicate his secret of manned flight until mankind became more virtuous.

Since 1759 there have been many other authors who have espoused the argument that scientists ought to cease work and suppress their findings as soon as it is clear that damage to their fellow men might ensue. I doubt if these arguments would have carried much weight in Dr Johnson's day, in a society that was largely rural and agricultural and for which the relatively feeble power of the human biped was augmented only by that of large mammals or that available from wind or falling water. Johnson's younger contemporaries such as James Watt, Abraham Darby and Matthew Boulton changed this situation irreversibly by applying knowledge, largely empirically gained, to invent the steam engine and iron and steel rails and wheels. The revolution they started gathered momentum over about one and a half centuries and transformed Western society into a primarily industrial and urban one.

Amongst the by-products of the Industrial Revolution were Victorian self-confidence and the establishment of colleges and institutions where science, engineering and technology could be taught and advanced in the sure and certain hope that the knowledge would further mankind's power over the animate and inanimate world, then deemed to be the only route to human material progress. But there was a darker side which soon became evident and was manifested by environmental pollution and consequent disease, social strain and sometimes discord, and struggles between imperialist powers for access to the cheap raw materials and the captive markets necessary to maintain growth in the manufacturing industries. It is arguable that neither the Industrial Revolution nor its outcomes would have been foreseen either by those pioneers who made it possible or the nation's political leaders.

Now the society created by the Indust-

rial Revolution is giving way to another, and the engine of this change is science. The principal advances in knowledge are twofold: first, the powerful combination of the analysis of operations into a series of questions demanding a simple yes or no answer with the developments in solid state physics to make the micro-chip possible; and, secondly, the understanding of the control and information systems in living cells. The former has led to the computer, which has greatly augmented human mental power, whilst the latter has far-reaching implications for medicine, agriculture and the efficient and cheap production of chemicals. It seems likely that the judgement of history will be that this Information Revolution will be much more significant than the Industrial Revolution. It certainly poses weightier questions, for the potential benefits are so much larger and so are the risks.

In the twentieth century scientists have shown themselves to be aware of the dilemma which this knowledge poses for them. Some, like the founding members of the American Association of Atomic Scientists 40 years or so ago, thought they must make a concerted effort to explain to the public what the implications of unlocking the power of the atomic nucleus were. In so doing they were adopting the view, later articulated by Dr John Platt, that scientists should be the cartographers of the future, laying out for their fellow citizens a map of what was possible so that human societies could decide on which destinations and routes they wished to take. Just over ten years ago, molecular biologists meeting at Asilomar seemingly echoed Dr Johnson's philosopher in accepting a voluntary moratorium on research in genetic engineering, a breathing space in which to consider the social implications. To go the whole way with Dr Johnson would be wrong, however. Quite apart from the impracticability of exercising the responsibility to suppress the findings of scientific work in a consistent way, the scientific estate, however much more knowledgeable than the rest of the population, has no right to take such decisions on behalf of society.

The plain facts are that the decisions about science policy must in the end be taken as part of the normal political process and that much of the scientific enterprise of any country must be managed by the government of the day, if only because it is the main paymaster. In the first chap-

ter of his book, Dr Maurice Goldsmith tells the reader that societies will not be equipped to adopt a sensible attitude to contemporary science and scientists, let alone optimize science policy, unless there exists a body of "science critics". The science critic is defined as "a public policy journalist, alerting us to the growing pains of future worlds through the day-to-day discoveries of the present". No one would disagree with this proposition, but I doubt whether it is a sufficient as well as a necessary condition; societies are more complex than that and we know too little about their workings.

Chapter 1 brings us to this conclusion via a great deal of verbiage and quotations from gurus such as C.P. Snow, C.H. Waddington, J.D. Bernal and Aldous Huxley. Delightful they are too but quite unnecessary to the argument. The remaining chapters, though less loaded with quotations, are refreshingly different. They contain facts and tell us a good deal about scientific journalism, how it works, its priesthood and prizes. They offer a good deal of practical advice to anyone thinking of taking up this profession and possibly, though I am in no position to judge, will be helpful to existing practitioners.

The second and longest chapter is a description of how the various media operate in the Western world, and will repay attention by scientists who so often fail to get their message across and to understand the adaptations they must make to be successful in communicating to the public. There follows a brief essay on science fiction, a subject in which I could never be interested, because it seemed to me that science itself was so much more interesting and called for much greater leaps of imagination. Next there are two succinct essays on school science education (largely in Britain) and on how the message may be handled in a Third World country. I greatly enjoyed both of them; the first because I knew something about the subject, the second because I did not and the confidence engendered by the sound and balanced presentation of the first spilled over and gave me confidence that the second must be of similar quality.

Finally Dr Goldsmith returns to his new breed of animal, the science critic, whom he sees not just as a reporter of science but as someone who makes value judgements about it and its place in society. But to do so such a person would have to be equipped with a broad synoptic view of science, be able to see the future and discern similarities, and be able to interpret and communicate science and uphold its integrity. I could not but help feel that this chapter described the remarkable science teacher I had at school 60 years ago, which in turn made me consider the problem of generating a supply of such remarkable people to perform this necessary function. This is

briefly touched on by Dr Goldsmith, but I am sure he would be the first to admit that the time when we shall have an adequate supply of these critic-communicators is far distant. The remaining 60 per cent of the book consists of two appendices, the first of which is concerned with some of the nuts and bolts of science journalism, and the second with examples of popular science writing.

I enjoyed reading this book and I think many others would too. The trouble is that it touches upon many topics, none of which is explored in any depth, and the main conclusions seem to be so miniscule.

Going into a trance

Anthony W. Clare

The Induction of Hypnosis. By William E. Edmonston, Jr. Wiley: 1986. Pp.432. £42.25, \$43.95.

THERE are those, most notably Leon Chertok, who argue that the therapeutic element in psychoanalysis, in so far as there is one, is suggestibility, and that psychoanalysis, through its intermediary the transference, has not disengaged from hypnosis but has merely changed the terminology. Freud himself declared in 1921 that "we still have no explanation of the nature of suggestion itself, that is to say of the conditions in which one is subject to its influence". It is indeed the case that ever since then many analysts have retained a lively interest in the subject of hypnosis.

Contemporary hypnotists stress the importance of eye fixation and the attention-rivetting techniques employed to bring about the hypnotic state. They do so with the same degree of purist vigour as analysts emphasize the crucial importance of free association and interpretation, and behaviourists insist on the essential therapeutic features of conditioning and de-conditioning. The real question, however, is the emotional aspects of the therapeutic relationship, the affective content of the procedures rather than the actual theoretical procedures themselves; yet it is this affective component of therapy, bound up as it is in notions such as suggestibility and susceptibility, which is poorly studied and indeed rarely considered.

The fact remains that it was the nineteenth-century developments in hypnotherapy that facilitated the growth of modern psychotherapy, and it does not seem too far-fetched to believe that in the unravelling of hypnosis lies the answer to the thorny question of the efficacy of psychotherapeutic procedures in general. Such issues are not the subject matter of Edmonston's book any more than they seem to be the concern of many psychotherapists. But the contents of the book

Glancing at it again after my first reading, it seems to me that it was probably written, if not in Dr Johnson's record-breaking time, then very quickly from pieces that had already been published or were lying about nearly finished on the author's desk. No criticism of Dr Goldsmith for that, but merely to say that whilst it is a good read it is not a great book. □

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make it abundantly plain that such a state of affairs cannot last.

If the author of this detailed text on the subject is to be believed, the history of hypnotic induction is the history of medicine. It is true that the induction of sleep has been a mainstay of therapeutic interventions since before Hippocrates. References to what sound like hypnotic trance states can be found in Vedic medicine, in the Papyrus Ebers of Egyptian medicine fifteen centuries before Christ and in the healing rituals of the Irish Druids before the arrival of St Patrick. Not surprisingly, in the revised history of hypnosis Mesmer ceases to be the pivotal figure he seemed and is deemed to be not so much a pioneer as a catalyst, giving hypnosis a vital nudge forward at an opportune time.

Edmonston devotes a great deal of space to a historical review of the role of hypnosis and to an enumeration of the many, varied and elaborate ways of inducing a hypnotic trance. But at times the list of treatments falling under the rubric of hypnosis becomes rather elastic. It is doubtful, for example, that the methods of Valentine Greatrakes, a contemporary of William Harvey, were truly hypnotic, given that he relied upon the laying on of hands and the smearing of his patients at St Bartholomew's Hospital with spittle!

The real issue in contemporary hypnosis is not its historical roots, nor its induction methods. It is the precise relationship between classical hypnosis, with its emphasis on the induction of a trance or sleep-like state, and the newer task-orientated forms of behaviour therapy with their emphasis on relaxation, concentration, the relief of anxiety and the induction of tranquillity. A related question is to do with the relationship between the analytic transference, so stressed within psychodynamic psychotherapy, and the notion of suggestibility which lies at the heart of hypnotic methods. These, however, are matters that are scarcely touched upon in this book. □

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Our lady of radium

H.W. Paul

Marie Curie: A Life. By Françoise Giroud. Translated by Lydia Davis. *Holmes & Meier, New York: 1986. Pp.291. \$34.50.*

ROENTGEN'S discovery of X rays in 1895 led directly to the discovery of radioactivity by Henri Becquerel. Unlike X rays, radioactivity did not excite scientists or the public; no lead-lined knickers were sold to protect prudent women from its scrutiny. Then, in 1898, Marie and Pierre Curie announced their discovery of polonium and radium, and a radium craze soon infected the world. The public had not been so interested in science since the publication in 1859 of Darwin's book on the origin of species.

Maria ("Manyad") Skłodowska, a polyglot positivistic Pole from Warsaw, went to the University of Paris in 1891 to study science. Polish nirvana: freedom from the Russian yoke, cheap education, even for women, the opportunity to work for the improvement of mankind through science, and freedom to discuss the Polish condition without the threat of arrest. Marie was a brilliant student and immediately attracted the attention of Sorbonnard mandarins such as Paul Appell, Edmond Bouty and Gabriel Lippmann.

In 1895 she married into the tribe of French physicists, a rather inbred group. The marriage changed Pierre Curie's career, for he was seduced into collaborating with Marie on the scientific obsession of the day, radioactivity. Working in a shed at the municipal school of industrial physics and chemistry, they made their famous discoveries. International fame led to success in Paris: Pierre was given a chair at the Sorbonne, to prevent his going to Geneva, and after his death in 1906 the chair went to Marie. The science faculty's radical precedent of opening its ranks to women was not followed by the Académie des Sciences, which narrowly preferred physicist Edouard Branly of the Institut Catholique—a minor triumph for Catholicism over republicanism. Nearly a century before, women interested in science had been excluded from the newly-created faculty on the grounds that men would be more interested in them than in the professors' lectures. That must rank as one of the world's worst academic administrative decisions. In her long career Marie Curie won two Nobel prizes, one, with Becquerel and Pierre, in physics, and one for herself alone in chemistry. Amongst the many virtues of the Third Republic (1871–1940) was that it made possible, if improbable, a career in science for women.

In this book, Françoise Giroud has given us a very touching portrait of Marie

Curie. The history of science is sometimes handled cavalierly and the political passages are no better: Bismarck's Polish policy is reduced to a misleading *boutade*, and Prussia is generously given five million instead of two-and-a-half million Poles. Yet there is a great deal of reliable information on science artfully woven into the biography.

Madame Giroud often goes to the heart of matters with rare skill. The cultural and especially the social contexts are also deftly sketched, with emphasis on the economic exploitation of women. Glimpses into the private lives of the austere Curies and their more sybaritic colleagues provide some relief from Marie's grim devotion to science. But Marie Curie is not famous because she knew Paderewski or visited Rodin's studio, or even for her love affair with Paul Langevin which is treated in exquisite detail by Madame Giroud. Langevin's wife made the scandal notoriously public, and Paul Appell, the desperate dean of the faculty of sciences, wanted to solve matters by encouraging Marie to return to Warsaw. The American physicist B.B. Boltwood, a collaborator of Rutherford's, was satisfied that he had been correct in classifying her as a detestable idiot, but generous American support for Marie's work indicates that his opinion was not widely held in the United States.

The work of the Curies gave French science an international attention it had not enjoyed since the national orchestration of Pasteur's genius. The glory was equally deserved, and dozens of foreign researchers came to work on radioactivity at the Radium Institute in the Latin Quarter's vast city of science. In 1925 Marie picked Fred Joliot as her laboratory assistant, and his subsequent marriage to her scientist daughter Irene produced

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

In collaboration — Marie and Pierre Curie.

another great team in French physics. Joliot went on to become the main figure in the development of nuclear energy in France. Nearly up to her death in 1934 Marie was a tireless international ambassador for French science and an effective propagandist at home, where Jean Perrin and his friends were preparing the ground for the post Second World War structures and functions of French science.

Madame Giroud's book is heavily dependent on works such as Eve Curie's study of her mother. But its dramatic style and theme of *Une Femme Honorable* (the French title) make it a satisfying night's reading. □

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Optimal ideas

Brian Charlesworth

Physiological Ecology of Animals: An Evolutionary Approach. By R.M. Sibly and P. Calow. Blackwell Scientific: 1986. Pp.179. Hbk £26, \$49; pbk £11.95, \$24.50.

OVER the past decade, the quantitative application of the concept that the properties of living organisms represent optimal solutions to specific design problems has been a growth industry. Richard Sibly and Peter Calow have been prominent exponents of this approach, and the aim of this book is to set out some principles of optimization theory, and to apply them to a series of problems in physiological ecology and life-history evolution. The basic procedure is to assume a plausible set of constraints acting upon the properties of

interest in the particular study, for example that an increase in the amount of effort devoted to reproduction at a given stage of the life-cycle leads to a decrease in the chance of survival to the next stage. Given a criterion by which to measure darwinian fitness, one then calculates the set of values of the properties that yield a maximum in fitness according to the specified constraints. It is then assumed that natural selection will have caused populations of organisms to evolve close to this optimal set. Their observed properties are compared with the predictions of the model, in an effort to test the model's validity.

This procedure is often criticized on the grounds that failure of an optimization model to fit the real world does not provoke its originator to abandon belief in natural selection as the main force guiding the evolution of the characteristics of interest; rather, it usually leads him to tinker with the details of the model. This criticism

is somewhat misplaced, because the aim of workers using these methods is not to test the general validity of the theory of natural selection, but to uncover the biological factors which may be of significance in moulding the action of selection on the traits in question. Furthermore, given the complexity of biological systems one would have to be wildly optimistic to expect an accurate fit between predicted and observed properties. So emphasis has often been placed on theoretical predictions of the consequences of variation in ecologically determined factors which modulate the ways in which the system responds to selection, and on comparisons of populations with appropriate differences in ecology to see if they differ (at least qualitatively) in the expected fashion. Thus, it is a stock-in-trade of life-history theory that species living in environments with high adult survival relative to juvenile survival should invest less in reproductive effort per breeding season, compared with otherwise similar species with low adult survival.

Sibly and Calow bring out these principles in a number of case studies, ranging from an analysis of the factors affecting the efficiency of guts, to strategies of growth and reproduction. The emphasis is on the development of optimization models, rather than detailed testing against the data; indeed they state that their aim is to stimulate experimentalists to provide the necessary data. They succeed in convincing one of the diversity of topics to which their approach can be applied, and the book will be of considerable use to biologists in this area.

The authors can, however, be criticized on several points. One is that they give little indication of previous contributions to life-history theory, a relatively mature area of enquiry. Instead, they present their own reformulations of classic models as though they were wholly original, and provide only scanty citation of the literature. This will not help beginners find their way. More importantly, they fail to mention alternatives to optimization methods. Thus, there is no reference to Williams's and Medawar's theories of senescence, which have an important bearing on life-history evolution. Little mention is made of the possible role of competition in moulding selection on growth and size, and Maynard Smith's Evolutionarily Stable Strategy method for handling the complications that arise in competitive situations is not discussed. While the authors' approach has considerable value, and they have every right to advocate it, their book cannot be said to offer a comprehensive survey of the methods needed to solve problems in evolutionary ecology. □

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Steps out of time

Eric Ashby

Walkers. By Miles Jebb. Constable, London: 1986. Pp.202. £10.95.

SIXTY years ago, devotees of the non-fiction book at bedtime were well supplied by a genre which is now rare: the essay. Contemporaries of Chesterton, Belloc and Aldous Huxley had plenty of entertainment awaiting them at the end of the day. Today there are very few competent practitioners in that style of writing. One can still enjoy the classical essayists — Charles Lamb for the light-hearted, Emerson for the sober-minded, and Montaigne and Bacon — but who is writing in that style now?

Well, Miles Jebb for one. His book, *Walkers*, is in the tradition of the whimsical nineteenth-century essayists. In eleven brief chapters Jebb writes about the various kinds of people who walk or have walked: pilgrims, athletes, intellectuals, tramps, rambles... I'll not disclose the whole list. The book is a compilation of anecdotes and episodes from history and fiction, told with the comfortable informality of after-dinner conversation, by a man with a well-stocked mind.

If you start to delve into the literature

about walking, it is surprising what turns up. Each of Jebb's eleven kinds of walker finds himself heir to certain conventions. The value of a pilgrimage may be enhanced by discomfort: "The Countess of Clare threw away her shoes as she started on a pilgrimage". Athlete-walkers are out to break records: Sean Maguire, who "in 1978 walked the 7327 miles from Yukon to Florida in three hundred and seven days"; and Sebastian Snow, who reckoned he had taken some 11,745,000 paces in his walk from the tip of South America to Panama City with a 60 lb pack on his back. Intellectual walkers are the most articulate (witness Hilaire Belloc's *Path to Rome*) and they often philosophize as they walk. Or they boast of a kind of muscular intellectualism: thus Leslie Stephen, when at Cambridge, walked to London (over 50 miles) in 12 hours to attend a dinner of the Alpine Club.

Tramps fall into two categories: those who walk because they have nowhere else

to go, and those who "walk away from convention". The former are inarticulate (though the poet W.H. Davies was an exception); the latter, such as Stevenson and Maxim Gorky, often become tramps in order to collect material for writing. Ramblers not only ramble; they combine to form pressure groups to lobby for conservation, and they sometimes flock together in group walking. They are somewhat querulous, having been driven off the roads by traffic, but we are indebted to them for their lobbying: in the United States, the Sierra Club has a fine record in defending the wilderness, and in Britain the Ramblers' Association has preserved many footpaths that would otherwise have been obliterated by farmers.

If you like crude and genial conversation about themes such as these, this is a bedtime book to savour. □

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Thought of biology

P.T. Saunders

Biophilosophy: Analytic and Holistic Perspectives. By Rolf Sattler. Springer-Verlag: 1986. Pp.284. Pbk DM66

ROLF Sattler is a biologist who believes that it is important to understand the fundamental presuppositions of biology and how they influence research and even our view of life itself. In *Biophilosophy* he sets out to introduce other biologists to these ideas.

I am in full sympathy with Sattler's aim, and he has gone some way towards achieving it. But there are reservations. The book seems to be intended as both an introductory text and a monograph, and rather falls between the two stools. Far too much material has been crammed into a text that also has to include elementary expositions of basic ideas. As a result, some explanations are marred by what are, for the beginner, digressions. At other times, Sattler rushes along at great speed; he deals with the issue of free will in just over a page. Many terms and ideas are mentioned so fleetingly that the reader not only is left uninformed about them but will not even be able to grasp how they are relevant to the discussion.

Often Sattler does no more than mention that a certain idea has been put forward and give some references. For example, he considers that the idea of species as individuals rather than classes leads to the scientific view of unity or oneness to which he devotes the last chapter. Yet he says very little in support of the idea, beyond mentioning the common gene pool and providing some references to "a critical

appraisal" of the concept. It would have been better to spend more time on those questions that he considers to be most important, and to compensate by omitting some other material altogether.

Biophilosophy, then, is not a satisfying book. The author seems to have tried to get down in print everything he has thought about, from plant morphology to Zen Buddhism. Further, aspects of the selection of material and the relative emphasis given to different topics mean that he has not fully assimilated all that he has learned into a coherent whole. On the other hand, if *Biophilosophy* loses by not having been written by a professional philosopher, it also gains from having been written by a working scientist to whom these ideas matter, and who sees many of the issues from the same point of view as his readers.

Far too few biologists are aware of the philosophical issues that underlie and influence what they do. Moreover, because so much of the philosophy of science is concerned with physics, not biology, it is not readily accessible to them. Biologists who read this book will become acquainted with many important questions, and, what is more, will have them explained in familiar terms — there are not many introductions to the philosophy of science that illustrate the basic concepts through plant morphology, instead of quantum mechanics. I even approve of reminding the reader that plant morphology and Zen Buddhism are both worth thinking about, and not necessarily in separate intellectual compartments. I only wish Sattler had tightened the whole thing up a bit. □

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Forty years of genetic recombination in bacteria

Between April and June 1946, Joshua Lederberg and Edward L. Tatum carried out a series of experiments that proved that bacteria can exchange their genes by sexual crossings. The experiments were reported in Nature just 40 years ago¹. In the following pair of articles, Joshua Lederberg first provides a personal reminiscence of the circumstances of the discovery and then, together with Harriet Zuckerman, considers it as a possible case of 'postmature' scientific discovery.

A fortieth anniversary reminiscence

Joshua Lederberg

IN September 1941, when I started as an undergraduate at Columbia University, the genetics of bacteria was still a no-man's-land between the disciplines of genetics and (medical) bacteriology. The question whether "bacteria have genes, like all other organisms" was still unanswered, indeed rarely asked. My own thoughts at that moment lay elsewhere. I looked forward to a career in medical research applying chemical analysis to problems like cancer and the malfunctions of the brain. Cytotoxicology then appeared to be the most promising approach to cell biochemistry. It was Frances J. Ryan (d. 1963) who turned my attention to the sharper tools of genetics.

Ryan had spent 1941–42 as a postdoctoral fellow at Stanford University, where he had met G. W. Beadle and E. L. Tatum (d. 1975), and had become fascinated with their recent invention of nutritional mutations in *Neurospora* as a tool for biochemical genetic analysis². Although working on a fungus like *Neurospora* did not go down smoothly in a Department of Zoology as at Columbia, where Ryan had accepted an instructorship, he established a laboratory to continue these studies. In January 1943 I was fortunate to get a job in his laboratory assisting in the preparation of media and handling of *Neurospora* cultures. Ryan's personal qualities as a teacher and the setting of serious research, discussion with him, other faculty members and graduate students in the department nourished my education as a scientist. On 1 July 1943, I was called to active duty in the United States Naval Reserve, and my further months at Columbia College alternated with spells of duty at the United States Naval Hospital, St Albans, Long Island. There, in the clinical parasitology laboratory, I had abundant opportunity to observe the life cycle of *Plasmodium viva*. This experience dramatized the sexual stages of the malaria parasite, which undoubtedly sensitized me to the possibility



Lederberg in 1945

of cryptic sexual stages in other microbes (perhaps even bacteria). In October 1944, I was reassigned to begin my studies at Columbia Medical School; but I continued working with Ryan at the Morning-side Heights campus.

Discovery

The important biological discovery of that year, by Avery, MacLeod and McCarty, was the identification of DNA as the substance responsible for the *Pneumococcus* transformation³. This phenomenon could be viewed as the transmission of a gene from one bacterial cell to another; but such an interpretation was inevitably clouded by the obscure understanding of bacterial genetics at the time. Avery's work, at the Rockefeller Institute in New York, was promptly communicated to Columbia biologists by Theodosius Dobzhansky (who visited Rockefeller) and by Alfred Mirsky (of the Rockefeller faculty) who was a close collaborator of Arthur Pollister in the Zoology Department. The work was the focus of widespread and critical discussion among the faculty and students. Mirsky was a vocal critic of the purported chemical identification of the transforming agent, while applauding the central importance of the work. For my own part, the transcendent

leap was simply the feasibility of knowing the chemistry of the gene. Whether this was DNA or protein would certainly be clarified quickly, provided the *Pneumococcus* transformation could be securely retained within the conceptual domain of gene transmission. I read the Avery, MacLeod and McCarty paper on 20 January 1945, prompted by Harriett Taylor (later Ephrussi-Taylor) a graduate student in Zoology who planned to pursue her postdoctoral studies with Avery. My excited response is recorded as ... "unlimited in its implications... Direct demonstration of the multiplication of transforming factor... Viruses are gene-type compounds."

At once, I thought of attempting similar transformations by DNA in *Neurospora*. This organism had a well understood life-cycle and genetic structure. The biochemical mutants opened up by Beadle and Tatum also allowed the efficient detection of nutritionally self-sufficient (prototrophic) forms, even if these were vanishingly rare. This would facilitate the assay of transformational events.

Between January and May, 1945, I shared this idea with Francis Ryan; in June, he invited me to work on the subject with him. To our dismay, we soon discovered that the leucine-minus *Neurospora* mutant would spontaneously revert to prototrophy⁴, leaving us with no reliable assay for the effect of DNA in mediating genetic change in *Neurospora*. Questions about the biology of transformation would remain inaccessible to conventional genetic analysis if bacteria lacked a sexual stage. But was it true that bacteria were asexual? Rene Dubos' monograph, *The Bacterial Cell*⁵, footnoted how inconclusive the claims were for or against any morphological exhibition of sexual union between bacterial cells.

My notes dated 8 July 1945 detail hypothetical experiments both to search for mating among *Monilia* (medically important yeast-like fungi) and to seek gene-

tic recombination in bacteria (by the protocol that later proved to be successful). These notes coincide with the beginning of my course in medical bacteriology. They were provoked by the contrast of the traditional teaching that bacteria were *Schizomycetes*, asexual primitive plants, with an appreciation of sexuality in yeast⁶, which was represented at Columbia by the graduate research work of Sol Spiegelman and Harriett Taylor.

Dubos⁷ cited many unclear, and two clear-cut negative results^{7,8} for sexuality in bacteria using genetic exchange methodology. But these two studies had no selective method for the detection of recombination and so would have overlooked the process had it occurred less often than perhaps once per thousand cells. With the use of a pair of nutritional mutants, say A^+B^- and A^-B^+ , one could plate out innumerable cells in a selective medium and find a single A^+B^+ recombinant. In early July, I began experiments along these lines. In the first instance I used a set of biochemical mutants in *Escherichia coli*, which I began to accumulate in Ryan's laboratory. To avoid the difficulty that had arisen in our *Neurospora* experiments, a spontaneous reversion from A^-B^+ to A^+B^+ , the strategy would be to use a pair of double mutants: $A^-B^+C^+D^+$ and $A^+B^-C^-D^-$. Sexual crossing should still generate $A^+B^+C^+D^+$ prototroph recombinants. These would be unlikely to arise by spontaneous reversions which, in theory, requires the coincidence of two rare events; $A^- \rightarrow A^+$ and $B^- \rightarrow B^+$. Much effort was devoted to control experiments to show that double reversions would follow this model, and so occur at a negligible frequency in the cultures handled separately. Thus the occurrence of prototrophs in the mixed cultures would be presumptive evidence of genetic recombination.

Long shot

Meanwhile at Stanford, Ed Tatum, whose doctoral training at Wisconsin had been in the biochemistry of bacteria, was returning to bacteria as experimental subjects, having published two papers on the production of biochemical mutants in *E. coli*⁹, including double mutants like those described here. During the summer of 1945 Francis Ryan learned that Tatum was leaving Stanford to set up a new programme in microbiology at Yale. He suggested that, rather than merely ask Tatum to share these new strains, I apply to work with him and get the further benefit of his detailed experience and general wisdom. Tatum agreed and suggested that I arrive in New Haven in late March, to give him time to set up his laboratory. He hinted that he had some similar ideas of his own, but never elaborated them. The arrangement suited him by leaving him free to complete his work on the biochemistry of

Experimental luck

1. We have learned¹² that *E. coli* strain K-12 itself was a remarkably lucky choice of experimental material: only about one in twenty randomly chosen strains of *E. coli* would have given positive results in experiments designed according to our protocols. In particular, strain B, which has become the standard material for work on bacteriophage, would have been stubbornly unfruitful. Tatum had acquired K-12 from the routine stock culture collection in Stanford's microbiology department when he sought an *E. coli* strain to use as a source of tryptophanase in work on tryptophan synthesis in *Neurospora*¹³. The same strain was then in hand when he set out to make single,

and then double mutants in *E. coli*⁹. In 1946, I was very much aware of strain specificities and was speculating about mating types (as in *Neurospora*). I have no way to say how many other strains would have been tried, or in how many combinations, had the June 1946 experiments not been successful.

2. An equally important piece of luck was that, the selected markers Thr (threonine) and Leu (leucine) are found almost at the origin of the *E. coli* chromosome map¹⁴. The cognoscenti will recognize that in a cross $B^-M^-T^+L^+F^+ \times B^+M^+T^-L^-F^-$, the configuration used in June 1946, these chromosome localizations offer almost a maximum yield of selectable recombinants. We were therefore led stepwise into the complexities of mapping.

Neurospora, perform the heavy administrative duties of his new programme, and still participate in the long-shot gamble of looking for bacterial sex.

It took about six weeks, from the first serious efforts at crossing in mid-April 1946, to establish well-controlled, positive results. These experiments could be done overnight, so the month of June allowed over a dozen repetitions, and the recruitment of almost a dozen genetic markers in different crosses. Besides the appearance of $A^+B^+C^+D^+$ prototrophs, it was important to show that additional unselected markers in the parent stocks would segregate and recombine freely in the prototrophic progeny. This result left little doubt as to the interpretation of the experiments.

An immediate opportunity for public announcement presented itself at the international Cold Spring Harbor Symposium in July. This was dedicated to the genetics of microorganisms, signalling the postwar resumption of major research in a field that had been invigorated by the new discoveries with *Neurospora*, phage, and the role of DNA in the *Pneumococcus* transformation. Tatum was already scheduled to talk about his work on *Neurospora*. We were granted a last-minute improvisation in the schedule to permit a brief discussion of our new results.

The discussion was lively. The most principled criticism came from Andre Lwoff who worried about cross-feeding of nutrients between the two strains without their having in fact exchanged genetic information. Having taken great pains to control this possibility, I felt that the indirect genetic evidence was quite conclusive. Fortunately, Max Zelle mediated the debate, and generously offered to advise and assist me in the direct isolation of single cells under the microscope. These subsequent observations did quiet remaining concerns of the group that Lwoff had assembled at the Pasteur Institute, including Jacques Monod, Francois Jacob and Elie Wollman, who were to make the most

extraordinary contributions to the further development of the field. The single cell methods were also useful in later investigations in several fields. A direct result of the Cold Spring Harbor meeting was the prompt ventilation of all the controversial issues. With a few understandable, but minor points of resistance, genetic recombination in bacteria was soon incorporated into the mainstream of the burgeoning research in molecular biology, and after another decade or so into the standard texts of bacteriology. It still took some years to work out the intimate details of crossing in *E. coli*; some, including the crucial question of the physical mechanism of DNA transfer between mating cells, are still obscure.

The public image of the scientific fraternity today has seldom been so problematic and the system cannot avoid putting a high premium on competition and self-assertion. We can recall with gratification how the personalities of Ryan¹⁰ and Tatum¹¹ exemplified norms of nurture, dignity, respect for others, and above all a regard for the advance of knowledge.

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Postmature scientific discovery?

Harriet Zuckerman and Joshua Lederberg

NEW scientific discoveries do not always flow directly from those made just before. Rather, several varieties of discontinuity can be identified in the growth of science. Premature discoveries are those that scientists do not attend to in a timely way, and are retrospectively described as having been "ahead of their time". These have been examined by Barber¹ and Stent². Here, we suggest that there are also postmature discoveries, those which, are judged retrospectively to have been 'delayed'. We analyse the arguments that the discovery of bacterial sex was postmature and take up the correlative questions of how the problem was identified, and why Lederberg and Tatum^{3,4} were likely candidates for making it when they did.

This paper draws on documents, published and private, and analyses by the sociologist-observer and the scientist-participant. Our dialectic procedure departs from most oral histories^{5,6}; first, the procedure was iterative: as new discussion raised further possibilities, we both searched for relevant documentation; and second, we both identified the underlying analytic questions and articulated tentative answers to them. We felt that personal reminiscence had to be validated by contemporary documents and other testimony as oral history and autobiography are prone to "unconscious falsification"⁷.

Continuities and discontinuities

Scientific growth, usually broadly incremental, can at important times be episodic and discontinuous. Premature discoveries, one conspicuous form of temporal discontinuity in science, are either passively neglected or actively resisted at the time they are made. Mendel's discovery of particulate inheritance in 1865, lost to view for thirty-five years, is the best-known historical case. Discoveries can be premature because they are conceptually misconnected with 'canonical knowledge'⁸, are made by an obscure discoverer, published in an obscure place, or are incompatible with prevailing religious and political doctrine. Barriers between disciplines imposed by specialization of inquiry also contribute to neglect or resistance^{1,8-10}. Although the character and sources of premature discoveries have received some analytical attention^{8,11}, the pattern of postmature discoveries has not been identified, much less systematically studied.

For a discovery to qualify as postmature, for it to evoke surprise from the pertinent scientific community that it was not made earlier, it must have three attributes. In retrospect, it must be judged to have been technically achievable at an

earlier time with methods then available. It must be judged to have been understandable, capable of being expressed in terms comprehensible to working scientists at the time, and its implications must have been capable of having been appreciated.

Both prematurity and postmaturity can be recognized only by retrospection. They differ in that prematurity is a matter of actual historical observation while postmaturity is a matter of retrospective conjecture. Such formulations would seem to smack of 'Whig History', the inclination, according to Butterfield, "to produce a story which is the ratification if not the glorification of the present"¹². But, they are designed to serve quite the contrary purpose. The ideas of prematurity and postmature discovery provide convenient handles for analysing discontinuities in the growth of scientific knowledge, and support a nonlinear and complex model of advancement in scientific understanding.

Postmature discoveries are not all of a piece. One class results from pre-emption of scientists' research attention. For example, Linus Pauling observed that there was "no reason why" he, himself, could not have discovered the alpha helix eleven years earlier than he actually did "after a few hours of work". In fact, he was preoccupied in the interval by other seemingly more important and feasible inquiries^{13,14}. Another class of postmature discoveries answer questions not previously recognized by scientists to be problematic. Certain assumptions, beliefs and images¹⁵ which are also indispensable for the organization of scientific thought can, in specific cases, impede perception of lines of inquiry. For example Weinberg notes that physicists neglected to pursue quantum field theory further in the 1930s because prevailing images, conceptual schemes and attitudes toward theory and empirical evidence stood in the way¹⁶. In our case study, both cognitive and social processes obstructed the thinking of scientists about recombination in bacteria.

Sources of neglect

Why was recombination in bacteria not perceived as problematic before 1946? How had asexuality in bacteria come to be an unquestioned 'truth' and how was that view perpetuated?

Before 1870, many believed that the different shapes bacteria assumed were varieties of the same organism, which changed under varying conditions. Indeed, the doctrine of polymorphism or bacterial plasticity became the basis for extravagant claims about variability through most of the nineteenth century.

By 1872, Ferdinand Cohn concluded that the various shapes bacteria took were not different forms of the same organism; they were monomorphic and did not change during their short lifetimes¹⁷. Yet reports of variation continued until 1881 when Robert Koch introduced a simple and effective means for growing pure cultures. Koch's pure-culture method, which became a symbol of modern bacteriology with its phobia of contamination, together with Cohn's doctrine of monomorphism rapidly changed bacteriologists' views about variation. The two were consolidated into what was called the Cohn-Koch Dogma, which discouraged for years the study of the problems of morphology, inheritance and variation in bacteria¹⁸.

Cohn was convinced that bacteria were primitive plants which could "only reproduce by asexual means" and in 1875 characterized all bacteria as *Schizomycetes* or 'fission fungi'. With every use of that label, bacteriologists were reminded that these organisms reproduced only by fission and that they were simple primitive plants, a tradition that had begun with Leeuwenhoek's first observation of bacteria in 1675. Labels, categories, nomenclature and taxonomies usually help to organize scientific thought but can also delay the reexamination of fallacious traditions, thus becoming self-fulfilling prophecies¹⁹. In the end, the emergence of medical microbiology as a science depended on the doctrinal base laid down by Cohn and the pure culture methods of Koch. Nonetheless, monomorphic doctrine, when strictly construed, threw out the baby of bacterial variation with the dirty bath water of contamination. It was widely assumed that observations of bacterial variation had to result from contamination. Bacteriologists took experiments involving variation to be error-prone and disreputable²⁰. Such experiments were to be avoided as having great procedural difficulty and little intellectual merit. With the strong incentives in science for avoiding problems notorious for leading to irreproducible results, very few scientists would elect to undertake them.

Bacteria occupied an ambiguous place in the hierarchy of living organisms. To many, these organisms appeared so primitive that they could not yet have evolved 'differentiated genes'. This image also reinforced the use of bacteria as exemplars of pre-genic levels of organization for physico-chemical analysis. Once such complex imagery becomes established, special provocation is needed to splinter away one or more of its elements.

Disciplinary emphases and the division

of labour among the sciences also diverted attention from the problem of bacterial sexuality. Bacteriologists were principally concerned with problems in medical pathology rather than issues like the biology of bacterial reproduction. Geneticists were no more interested in bacterial reproduction than bacteriologists. They were occupied with larger organisms in which the products of crossing were readily observed. Thus, disciplinary division of labour and the careful choice of organisms for inquiry, both generally conducive to the development of scientific knowledge, contributed in this instance to neglect of bacterial recombination. It has been argued, however, that 'disciplinary dogmatism' and 'disciplinary monopoly' have only rarely impeded the development and diffusion of scientific innovation²¹.

Members of the Delft School of Microbiology, in the early part of this century, did bridge the gap between bacteriology and genetics. Clearly separating themselves from the medical bacteriologists who maligned bacteria, they believed that progress in fundamental microbiology depended on people who 'loved' microbes²². Martinus Beijerinck, the main figure in the group, seems now to have been the most likely candidate for investigating bacterial sex. He rejected prevailing dogma on bacterial invariability, promptly cited deVries' finding on plant mutations and offered some of the first coherent challenges to strict monomorphism²³. He also developed 'enrichment culture' methods, forerunners of the selective techniques used later in discovering bacterial recombination. Moreover, he was better informed than most microbiologists about work on plant hybridization which would have been useful in planning any investigation of sex in bacteria. Beijerinck and the Delft School were likely candidates for investigating bacterial sex, but they did not. In fact, Beijerinck strongly supported the Cohnian dogma of Schizomycetes. Thus the problem of sexual recombination still fell between disciplinary schools²⁴.

Significance of bacterial sex

By the 1930s, developments were under way that led biologists to reexamine how bacteria related to other forms of life and whether bacteria really had genes. Important among these developments was the unification in biological thought of Mendelian genetics, quantitative population theory and darwinian evolution, particularly the notion of species being Mendelian breeding populations or isolated gene pools. The idea that sexuality was, itself, an evolved genetic system proved particularly provocative, with illustrations drawn from simple and complex plant life. Dobzhansky's monograph, "*Genetics and the Origin of Species*"²⁵, was widely read as the definitive reinterpretation of darwinian

theory of evolution and focused interest on the details of breeding systems as the key to understanding evolutionary development. This, in turn, sharpened interest in understanding the evolution of organisms, like bacteria, believed to be devoid of sexual mechanisms.

The biochemical analysis of microbial nutrition, especially by Knight and Lwoff¹⁸, was another major impetus to reexamining the relationship of bacteria to other forms of life. In particular, the discoveries that the biochemistry of microbes paralleled in many details that of higher organisms inspired Beadle and Tatum's work on *Neurospora* in 1941²⁶. They showed *Neurospora*'s usefulness as a research material for studying the genetic control of an organism's development through the encoding of specific enzymes, known as the 'one-gene-one-enzyme' hypothesis. This marriage of biochemistry and genetics and particular significance for the Lederberg-Tatum work^{27,28}.

There was also renewed speculative interest in a biochemical theory of the origin of life. "*The Origin of Life on Earth*"²⁹ by the Russian biochemist, Oparin, became available in English in 1944, as did "*What is Life?*"³⁰ by the physicist, Schrödinger. Both focused attention on questions that demanded the integration of the biology of viruses and microbes with the more traditional biology of plants and animals.

The connections between these independent developments were not always apparent at the time. But one event did call attention to their common message; the discovery by Avery, MacLeod and McCarty in 1944 which identified DNA as the transforming principle that changed rough non-pathogenic pneumococci into smooth virulent ones.

The scientific significance of that discovery has been examined in detail³²⁻³⁸. For our purposes, it highlighted two important questions: what was the structure of bacterial genes and how were they transferred? Thus the work by Avery *et al.* made the question of bacterial sex newly consequential. Dubos³⁹ makes it clear that had sexual reproduction been observed, it would have been understood and appreciated. But bacteria were so widely assumed not to reproduce sexually that no one considered this problem to be important. Dogma prevailed over focused curiosity.

Structural contexts

In retrospect, Lederberg's position in the communication network and his not yet having a career in science seem consequential for his identifying the problem of bacterial sex, for his developing a method for its investigation and for his being in a position to do the research. Tatum was led to the problem independently for somewhat different reasons²⁷. Lederberg was unenthusiastic about classical genetics when he arrived at Columbia College in

1941. His interest in "understand[ing] the chemical nature of life" led him to spend much of the next four years studying chemistry, cytology and physiological embryology. But he was not ignorant of classical genetics and the Columbia biologists were well connected with the New York network of scientific communication about genetics. Dobzhansky was a central figure. Arthur Pollister was in close touch with researchers at the Rockefeller Institute. Alfred Mirsky worked at both institutions. Lederberg not only learned quickly about the neo-darwinian developments described earlier but he also heard about the work of Avery *et al.* from Mirsky and promptly read their paper. If the Avery *et al.* work sharpened Lederberg's interest in bacterial reproduction, Dubos' review of the inconclusiveness of evidence on sexual reproduction sharpened his scepticism; the cognitive and structural elements were coalescing.

In Lederberg's second year at Columbia he met Francis Ryan, an assistant professor, who had just completed a post-doctoral fellowship at Stanford with Beadle and Tatum. It was Ryan who first told him about the work on biochemical genetics and who persuaded him that chemistry and genetics were not as far apart as he had thought²⁷. It was also Ryan who generously provided Lederberg with laboratory facilities, catalysed his association with Tatum, and, most importantly, encouraged, educated and socialized him as a scientist. Columbia provided Lederberg with a multifaceted and advantageous structural context for his scientific development and for the initiation of a high-risk, high-stakes research programme.

Lederberg's plan for research was well worked out by July 1945, when he was a second-year student at Columbia Medical School but continued to work in Ryan's laboratory. The research might have been pursued at Columbia, but Ryan and Lederberg both recognized that an association with Tatum would be valuable. In particular, his experience in microbial biochemistry could help broaden Lederberg's education beyond the opportunities available on Morningside Heights. Furthermore, Tatum, then in the process of moving to Yale, was rapidly becoming recognized as a scientific leader. He could provide Lederberg with better access not only to information, research materials and fellowship support, but also to the invisible college of the emerging scientific discipline of biochemical genetics. The impact of such informal ties between investigators on the directions and pace of scientific research has yet to be properly investigated.

Lederberg's status as a medical student was less an obstacle to his investigating bacterial sex than might be supposed. Though much of his time was spent on course work, he was not subject to the

constraints that apply in the early years of study for the PhD. He did not, like ordinary graduate students, have to choose a research problem that would be suitable for a thesis and publication. Being marginal⁴⁰ to the biological research enterprise, he could afford to take on a high-risk problem. The search for bacterial sex was definitely high-risk; it was not one likely to produce useful and publishable findings. After all, not observing bacterial recombination would scarcely demonstrate that it did not exist. The risk of a negative finding using *E. coli* is now known precisely; bacterial recombination being observed in only five per cent of all strains with the techniques used in 1946.

For a different set of reasons, Tatum could also afford research on a high-risk problem at the time. He had a variety of projects in process in his laboratory and could manage to take a long-shot experiment that required little time and little money. For both men, bacterial recombination was a good gamble; failure would have low marginal costs for each but promised large if prospectively improbable returns. High-risk investigations are not equally feasible for all scientists. They fall to the comparatively well-established or to those who are marginal as Lederberg was in 1946. Those who solve high-risk problems, having chosen them in the first place, may more often come from the ranks of the well established than from neophytes thus contributing to the accumulation of advantage⁴¹. Risk-taking in science is a matter not only of psychological daring but also of position in the social structure^{42,43}.

After a brief correspondence, Tatum invited Lederberg to work at Yale. He arrived in March 1946; genetic recombination in *E. coli* was experimentally observed early in May. The results were so arresting that Tatum arranged for Lederberg to present them at the Cold Spring Harbor Symposium to be held in July. The publications which followed^{34,43} did not merely describe the results of the initial laboratory investigation. They are the product of critical discussion of those results at that meeting and of follow-up experiments done immediately afterwards²⁷. The dynamics or organized scepticism in science⁴⁴ can be observed in the records of that meeting and later in responses to the papers announcing the discovery. Even as first published, discoveries are not simply reports of events initially observed in the laboratory^{45,46} but often are also the outcomes of exchange between contributors and their critics. Treating scientific contributions as the results of inquiry, criticism and subsequent work makes problematic the custom of designating this or that scientist as the exclusive contributor and focusses attention on the operation of organized scepticism and its effect on shaping the meaning and assessment of those contributions.

Conclusions

Was the investigation of sexual recombination in bacteria postmature, that is, conducted significantly later than it could have been? The problem was obscured for decades by the Cohn-Koch dogma of monomorphism and the conviction that bacterial variation resulted only from contamination. This was so even for Beijerinck and members of the Delft School who did not subscribe to strict monomorphism, knew how to mark microbial strains by their fermentative and nutritional characteristics (the basis of Lederberg's design), knew about Mendelian segregation in plants, and might have appreciated the significance of sexual recombination in bacteria were it observed. But, they were committed to the view that bacteria reproduced only by fission and did not consider the phenomenon problematic. In principle, the investigation was technically feasible by 1908, as demonstrated by Browning's⁴⁷ use of drug resistance as a selective marker, an early anticipation of the Lederberg-Tatum work. But Browning dealt with a different organism, reported a negative result, and used terminology not readily transferable to the case of bacterial recombination. In the 1930s, bacterial sex was still viewed as unlikely, even as a disputable idea. Yet had it been demonstrated experimentally, it would have been understood and appreciated by geneticists and possibly even by bacteriologists.

This case study suggests that problem-identification and selection in science have features deserving further analysis. First, the solutions to two classes of problems are apt to be postmature: those which do not survive competition for scientists' attention when they first appear because they seem insignificant, unfeasible or both and those which are obscured by prevailing cognitive commitments or have no socially and cognitively defined disciplinary home. Second, in calculating the probable returns on selecting problems for investigation, scientists assess the likelihood of error and this contributes to the continuing neglect of certain problems that have a history of being error-prone. Third, the feasibility of addressing high-risk problems in science and so of making major advances in this way is not equal for all investigators; they are left largely to the well-established who can afford them and to others who have a smaller stake, for structural reasons, in their immediate record of publication. What scientists define as problematic and worthy of investigation are the products of interactions between cognitive and social processes.

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Helium isotopes in sedimentary basins

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Measurements of helium isotopes in the ground waters and natural gases of sedimentary basins reveal that some possess a major component of mantle-derived helium, apparently related to their mechanism of formation. Neogene basins formed by loading do not involve introduction of significant mantle helium, whereas those formed by extension do.

MANTLE degassing occurs principally through young oceanic lithosphere, and to a lesser extent through tectonically active portions of continental crust¹⁻³. The phenomenon is particularly well documented for mantle-derived helium, which contains more ³He than crustal helium of purely radiogenic derivation. Stable parts of the continents are essentially impermeable to mantle volatiles, and helium from even the deepest boreholes has an isotopic composition characteristic of radiogenic production. But the formation of sedimentary basins involves deformation of the continental lithosphere which, depending on the processes involved, may or may not allow transient transfer of mantle-derived fluids into the crust. Here we suggest that the isotopic composition of helium in the ground waters and natural gases sampled from sedimentary basins may indicate the processes that acted during their formation.

Sedimentary basins may develop in different tectonic settings, and often the mechanisms involved in their formation remain poorly understood. Studies of the rates of basin subsidence, and the spatial and temporal variations of these rates, have shown that some basins have formed largely through thermal subsidence, whereas others appear to result from tectonic loading of the crust. Various explanations have been offered for basin formation through thermal subsidence⁴⁻⁷: one involves extension (or boudinage) of the lithosphere and upwelling of asthenosphere within the stretched zone⁶; another involves stretching of the lithosphere without thinning, extension being accommodated by the emplacement of mantle-derived dykes⁷. These are not mutually exclusive alternatives, and elements of both could be present in the development of any one basin. The North Sea basin provides a good example of thermal subsidence^{8,9}, and the demonstration of crustal thinning as well as aspects of the early subsidence history have suggested that this basin developed largely through lithospheric stretching.

When lithospheric stretching is accompanied by passive uprise of the asthenosphere, decompression may produce some melting¹⁰, and mantle volatiles may be released into the crust. When coupled with the elevated temperatures expected in the thinned crust⁶, such processes may facilitate the transport of mantle helium to shallow crustal levels. The dyke emplacement model for lithospheric extension⁷ could involve the release of mantle-derived volatiles from basaltic melts emplaced into the crust beneath the developing sedimentary basin.

Loading basins form where the crust is depressed adjacent to a tectonically imposed load, such as a stack of thrust sheets. Mantle melting is not expected to accompany the development of such basins, and enhanced transfer of mantle-derived fluids into the crust is not anticipated. The Molasse and East Carpathian basins of the Alpine chain and many of the basins east of the Canadian Rockies are examples of loading basins.

Helium isotope results for sedimentary basins

Helium isotope data are available for ground-water and natural gas samples from a number of sedimentary basins of contrasting

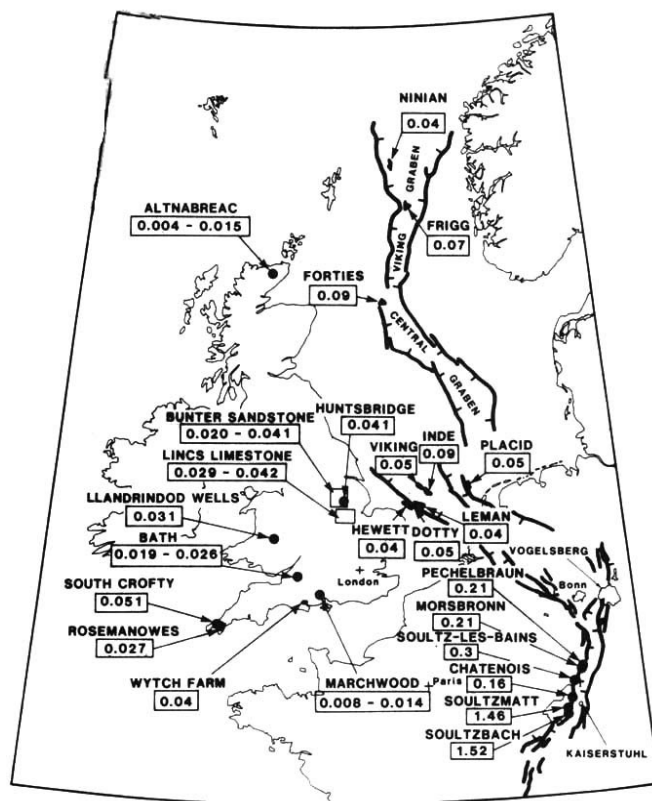
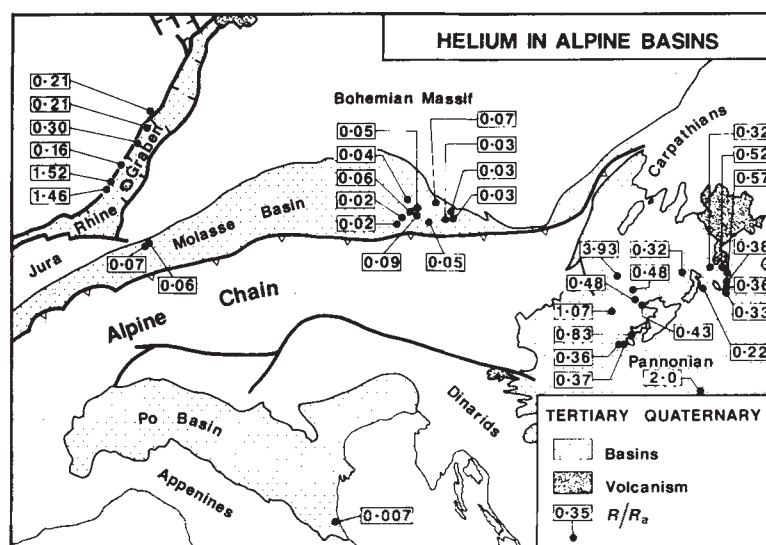


Fig. 1 Isotopic composition of helium in ground waters and natural gases in the mainland United Kingdom, North Sea and Rhinegraben. The values shown are for R/R_a , the ratio of the ³He/⁴He ratio of the sample (R) to that of the atmosphere (R_a). Natural gas samples from the North Sea basin have R/R_a values close to the expected radiogenic production ratio. A substantial mantle-derived helium component is present in samples from the southern part of the Rhinegraben. Data from ref. 12 and unpublished work.

age and style^{2,11-13} (Figs 1-3). The Molasse basin north of the Alps, the Pannonian basin of Hungary, the Rhinegraben and the Niigata basin of western Honshu (Japan) are used as examples of young (Neogene) sedimentary basins; the Mesozoic and Palaeogene North Sea basin and the Palaeozoic Hugoton-Panhandle gas fields provide information on older structures. Of these, only the Molasse basin is clearly formed by loading. Samples from the Niigata basin¹¹, the North Sea¹² and the Hugoton-Panhandle¹³ are all from producing gas wells; samples from the Rhinegraben¹² and from the Molasse and Pannonian basins (Fig. 2) were collected from producing water wells, and from deep-source springs. The two sampling modes each provide representative samples of the local crustal helium; in many cases

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Fig. 2 R/R_a values for ground waters from Neogene basins. Values for the Molasse basin (refs 30, 31 and W. Balderer, personal communication) show little evidence of the presence of mantle-derived helium. In contrast, the Pannonian basin samples³² all contain a mantle component.



it has been demonstrated that natural gases and formation waters from a single basin have comparable $^3\text{He}/^4\text{He}$ ratios¹⁴⁻¹⁶. The $^3\text{He}/^4\text{He}$ ratios (R) for samples from the various basins have been normalized to the atmospheric $^3\text{He}/^4\text{He}$ ratio ($R_a = 1.41 \times 10^{-6}$) and are presented in Figs 1 and 2.

Figure 3 summarizes the available data, which range from $0.02 R_a$ to $6.1 R_a$. Interpretation of the two extremes of the histogram is straightforward: values $>4 R_a$ indicate a substantial mantle helium component ($\geq 50\%$), whereas values close to $0.02 R_a$ indicate such low ^3He abundances that no mantle helium component is recognizable. The isotopic composition of radiogenic crustal He is controlled essentially by the abundance of lithium. ^4He is generated by the $^{238,235}\text{U}$, ^{232}Th decay series, whereas ^3He is derived from ^6Li by an (n, α) reaction. The composition of radiogenic helium may vary locally within a rock, reflecting the distribution of uranium, thorium and lithium between different phases and on different length scales. This problem has been examined in some detail¹⁶⁻¹⁹ and it appears that the composition of helium released from rocks is averaged over many possible generation sites. As helium samples are representative of relatively large ground-water or natural gas systems, for normal crustal lithologies radiogenic helium R/R_a is expected to average ≤ 0.04 , which is about the upper limit for water samples collected from different parts of the stable United Kingdom crust (refs 12, 20 and unpublished data). Although local departures may arise from anomalously lithium-rich lithologies, R/R_a values >0.08 are interpreted here as being mixtures of radiogenic helium ($R/R_a \approx 0.04$) and mantle helium ($6 < R/R_a < 10$). Anthropogenic ^3He (derived from bomb tritium) can be present only in very young (post-1950) ground-water components and needs to be considered only in the case of Molasse basin waters. The other fluids analysed either have helium contents too high for their composition to have been significantly affected by bomb tritium, or are demonstrably too old to be modified in this way, or both.

Neogene basins. The Niigata, Molasse and Pannonian basins began to form in the latest Palaeogene or early Neogene. The Niigata basin is an epicontinental back-arc basin behind the active northern Honshu volcanic arc. Subsidence began at ~ 23 Myr BP, and a thick sequence of volcanic and volcanoclastic rocks was deposited on a basement of Palaeozoic metamorphic rocks and Cretaceous granites. Volcanic input to the basin has waned since the end of the Miocene (6 Myr BP), although clastic sedimentation and deformation have continued to the present day.

Unlike the Niigata basin, whose development accompanied the opening of the Japan Sea²¹, the Pannonian basin is entirely intra-continental. It truncates many of the thrust units of the Alpine belt, and subsidence is associated with extensional and

strike-slip faulting, although the broader tectonic setting is not entirely clear^{22,23}. Volcanism within the Pannonian basin continued until the end of the Pliocene. The southern Rhinegraben is a narrow intra-cratonic rift which has been accumulating sediment since the Palaeogene. The region is seismically active today²⁴, and Tertiary volcanism continued until 11 Myr BP. The Molasse basin, in contrast, is a classic loading basin formed in the late Palaeogene and Neogene, and is the site of substantial Miocene deposition. The basin owes its origin to northward transport of the Upper East Alpine nappes and consequent loading of the Alpine foreland.

R/R_a values for helium from the Pannonian basin range from 0.22 to 3.9 and indicate the presence of a mantle-derived component ranging from 2.5 to 50%. The values for the Rhinegraben are similar but a little lower, (0.21–1.3) R_a . Those from the Niigata basin, however, are in some cases as high as $6.1 R_a$, indicating that locally the crust is completely swamped with mantle-derived helium. The Molasse basin helium has R/R_a values of 0.02–0.09, and nearly all lie within the anticipated range for radiogenic helium generated within the crust. The two highest values (>0.07) have the largest air corrections and the presence of anthropogenic ^3He is suspected; if this explanation of the high values is incorrect, the maximum amount of mantle helium in the Molasse basin samples is 0.9%. The Molasse basin shows no evidence for either volcanic activity contemporaneous with basin development nor evidence for crustal thinning. For these four young basins, the presence or absence of mantle helium correlates with tectonic setting.

Older basins. We now consider two older basins. The development of the North Sea basin began with two phases of rifting, in the late Carboniferous (~ 300 Myr) and Triassic (210–250 Myr), followed by Mesozoic deposition in fault-controlled extensional troughs. During the Tertiary and Quaternary there was broad regional subsidence, with the centre of the basin subsiding more than the margins. R/R_a values for helium from major North Sea gas fields and from the adjacent mainland United Kingdom ground waters (Fig. 1) range from 0.04 to 0.09. Contamination of these samples by anthropogenic ^3He is unlikely. Of the three samples with $R/R_a > 0.07$, two are from fields adjacent to bounding faults of the Central and Viking grabens. These relationships have been discussed in detail by Hooker *et al.*¹², who pointed out that the North Sea gas reservoirs are dominated by crustally produced radiogenic helium. The weak mantle signature which is locally present could have been acquired either at the time of hydrocarbon migration, or some time later by the leakage of mantle-derived helium upwards along major faults.

The Hugoton–Panhandle region of the south-central United

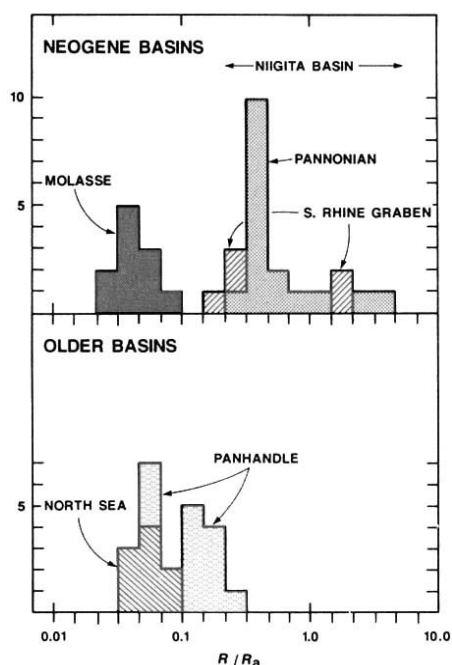


Fig. 3 Histograms of helium isotope compositions in Neogene and older sedimentary basins. Data sources as for Figs 1 and 2, and from ref. 11 for the Niigata basin. Note that the plotting intervals on the horizontal axis are unequal because the data are presented on a logarithmic scale.

States is of particular interest because it is one of the largest accumulations of natural gas discovered, and has helium contents of up to 2%. The gas reservoirs are in rocks of uncertain tectonic setting. The southern 'Panhandle' part of the reservoir is draped over a late Precambrian and early Palaeozoic basement high, the Amarillo uplift, and the northern 'Hugoton' fields are situated in sediments of the western margin of the Palaeozoic Anadarko Basin. The early history of the region appears to involve an early and rapid subsidence (from 525 to 500 Myr) with little accompanying volcanism, followed by an extended period of sedimentation until ~435 Myr (refs 25, 26).

R/R_a values for Panhandle gases vary from 0.05 to 0.21, and all but one of the thirteen samples analysed by Hill *et al.*¹³ show a clear mantle-derived component which reaches a maximum of ~2%. It is not known when the ^3He signature was acquired, and the helium is not necessarily residual from the time of basin formation¹³, but could be a recent or contemporary feature associated with igneous activity beneath the northern part of the field.

Discussion

Present knowledge of the processes that control the release of mantle volatiles and their ascent through the crust is extremely rudimentary. It is clear that igneous activity is very effective in this way, and almost everywhere that measurements have been made on gases issuing from fumaroles, volcanic vents or hot springs associated with volcanic activity, the presence of mantle helium has been detected. The three areas described above that have strongest mantle signatures, the Niigata and Pannonian basins and the Rhinegraben, have been the loci of Palaeogene and/or Neogene igneous activity and it could be suggested that degassing of helium from the mantle occurs only in association with melting and therefore with igneous activity. We now consider this possibility further, in the context of sedimentary basin formation.

For thermal subsidence models of sedimentary basin formation, the introduction of mantle melts into the crust during the extensional phase is an explicit feature of the dyke injection model and a possible consequence of asthenospheric decom-

pression in stretching models. Mantle helium would accompany melts into the crust, and could reach the upper crust before the maturation and migration of hydrocarbons.

Of the extensional basins that we have considered, three, the Pannonian basin, the Rhinegraben and the Niigata basin, have been active in the Neogene and are active to varying extents today. All have mantle helium in their ground waters, which presumably reflects degassing of helium recently derived from the mantle in each of these areas. Ground-water helium is likely to be lost to the atmosphere relatively quickly, and once degassing ends there may be no indication of the former presence of mantle volatiles in these areas, unless samples of crustal fluids are trapped and preserved in some way. Torgersen and Clarke²⁷ have recently argued, from helium concentrations of in ground waters, that degassing of helium from the continental crust is essentially a steady-state process.

The North Sea is essentially a Mesozoic basin in which slow subsidence in the axial zone continues today, whereas the Panhandle basin is of Palaeozoic age and has been inactive during the latter part of the Mesozoic and the Tertiary. The evidence for mantle volatiles in both cases comes from gases trapped in hydrocarbon reservoirs. The question in both cases is: when was the mantle gas introduced? If the release of mantle gases associated with basin formation ends before the development of traps, no gases are likely to be preserved. The persistence of a mantle helium signature in a sedimentary basin after its formation clearly depends on how long the mantle supply continues, and on the pathways followed by the mantle fluid in the crust. At the two extremes will be the gas trapped in the 'tight' reservoir, with the possibility of long retention, and the gas entrained in ground water that makes its way rapidly to the surface to be lost to the atmosphere. The decay times associated with these two extremes could range from several hundreds to less than tens of millions of years. In the former case the signature will also weaken with time through dilution with locally produced radiogenic crustal helium.

In the North Sea, hydrocarbon migration seems in some cases to have occurred nearly 150 Myr after the main extensional phase of basin development. If the extensional phase was accompanied by emplacement of mantle-derived melts, then the general absence of mantle ^3He in the basin today would require that helium be effectively lost in ≤ 100 Myr. The presence of mantle ^3He in the much older Hugoton-Panhandle rocks must imply either long-term retention from the Palaeozoic and therefore a quite different effective diffusivity structure for the crust compared with that of the North Sea, or alternatively a more recent input of mantle-derived gas.

In the case of the North Sea we note that, away from the Central Graben, all well gases analysed from the basin, and all groundwaters from its landward extension, show only radiogenic crustal helium. The only sign of mantle helium is a weak signature found along the distant northward extension of the active Rhinegraben faulting system into the North Sea, and it is tempting to suppose that there was a small amount of a localized mantle gas leakage at its northern end.

It is not clear whether the ^3He -rich helium is derived from the asthenosphere or the lithosphere. The convecting upper mantle, as sampled at spreading-ridge axes, is a well-mixed reservoir for helium with $6 < R/R_a < 10$, but the continental lithospheric mantle is also known to contain helium. Xenoliths of continental lithospheric mantle material, brought to the surface by recent volcanic activity, contain helium with a range of R/R_a values similar to those characterizing mid-ocean-ridge basalts (MORB)²⁸; however, it is not known whether helium of such a composition is to be found everywhere in continental lithospheric mantle. Helium in fluids from a wide range of circum-Pacific volcanoes has a relatively restricted compositional range around $R/R_a = 6$, and is interpreted as being a mixture of MORB-type helium from mantle beneath the volcanic arc and radiogenic helium derived from the subducted crust²⁹.

In the absence of evidence to the contrary, we assume that mantle helium is transported into the crust in melts, from which it subsequently degasses, and that melting occurs principally in the hotter asthenospheric material which undergoes decompression beneath at least some sedimentary basins formed by thermal subsidence. Note, however, that non-magmatic fluids of mantle origin could satisfy the helium observations equally well.

In summary, it appears that the presence or absence of mantle-derived ^3He at shallow levels in Neogene sedimentary basins relates to their mechanism of formation. The presence of significant amounts of mantle helium in the Pannonian and Niigata basins and the Rhinegraben is related to the introduction and degassing of mantle-derived fluids produced as an integral part of the extensional process. Extension by dyke injection cannot be easily distinguished from extension by thinning, although

some resolution may ultimately be possible because of predicted differences in the amounts of melt introduced. The Molasse basin, a classic loading basin, has developed without the introduction of identified mantle-derived melts or helium. The interpretation of the helium isotope composition in sedimentary basins whose development began in the Palaeozoic or Mesozoic, such as the Hugoton-Panhandle and North Sea basins, is less straightforward and requires some knowledge of the time required for the loss of helium in crustal regions undergoing basin development. Both of these basins have probably received late additions of mantle volatiles. Parts of this work were carried out under a research contract with the European Community, no. EN3G.004.UK(H). The ideas presented in this paper have been discussed with many colleagues, for whose comments we are extremely grateful.

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The c-erb-A protein is a high-affinity receptor for thyroid hormone

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Hormone binding and localization of the c-erb-A protein suggest that it is a receptor for thyroid hormone, a nuclear protein that binds to DNA and activates transcription. In contrast, the product of the viral oncogene v-erb-A is defective in binding the hormone but is still located in the nucleus.

THE avian v-erb-A oncogene does not by itself cause tumours in animals, but enhances the erythroblast-transforming potential of primary oncogenes, such as v-erb-B or other sarcoma-inducing oncogenes^{1,2}. Two specific effects of v-erb-A have been characterized. First, erythroblasts transformed by the primary oncogene alone spontaneously differentiate into erythrocytes, and v-erb-A completely inhibits this maturation. Second, v-erb-A allows transformed erythroblasts to propagate in media with wide ranges in pH and salt concentration^{1–3}. These observations suggest that v-erb-A in erythroid cells affects ion transport systems and regulatory factors of differentiation³.

It was recently demonstrated that the v-erb-A-specific part of the p75^{gag-v-erb-A} protein (encoded by the avian erythroblastosis virus, AEV-ES4) bears homology to receptors for steroid hormones^{4–7}, suggesting that the effects of v-erb-A could be due to direct transcriptional deregulation of genes responsible for the

aforementioned effects³. To understand these processes better we have isolated and characterized complementary DNA clones of the cellular erb-A gene as well as their proteins, and demonstrate that the c-erb-A protein has biochemical properties very similar to those of the receptor for the thyroid hormones triiodothyronine (T₃) and thyroxine (T₄) (see ref. 8 for review).

Cloning and sequencing of cDNAs for c-erb-A

The avian c-erb-A gene is transcribed into two messenger RNAs, 4.5 and 3 kilobases (kb) long, that are present at low levels (5–10 copies per cell) in many adult and embryonic tissues (ref. 9 and B.V., unpublished data). We therefore screened with a v-erb-A probe a chicken embryonic cDNA library prepared in λ bacteriophage gt11 (refs 10, 11). Ten positive clones were isolated from the 10⁶ plaques screened, and two (clones F1 and U1), that hybridized to probes representing 5' and 3' domains in v-erb-A, were subjected to nucleotide sequence analysis. Figure 1 shows the presence of a long open reading frame of 1,224 nucleotides homologous to v-erb-A, suggesting a relative

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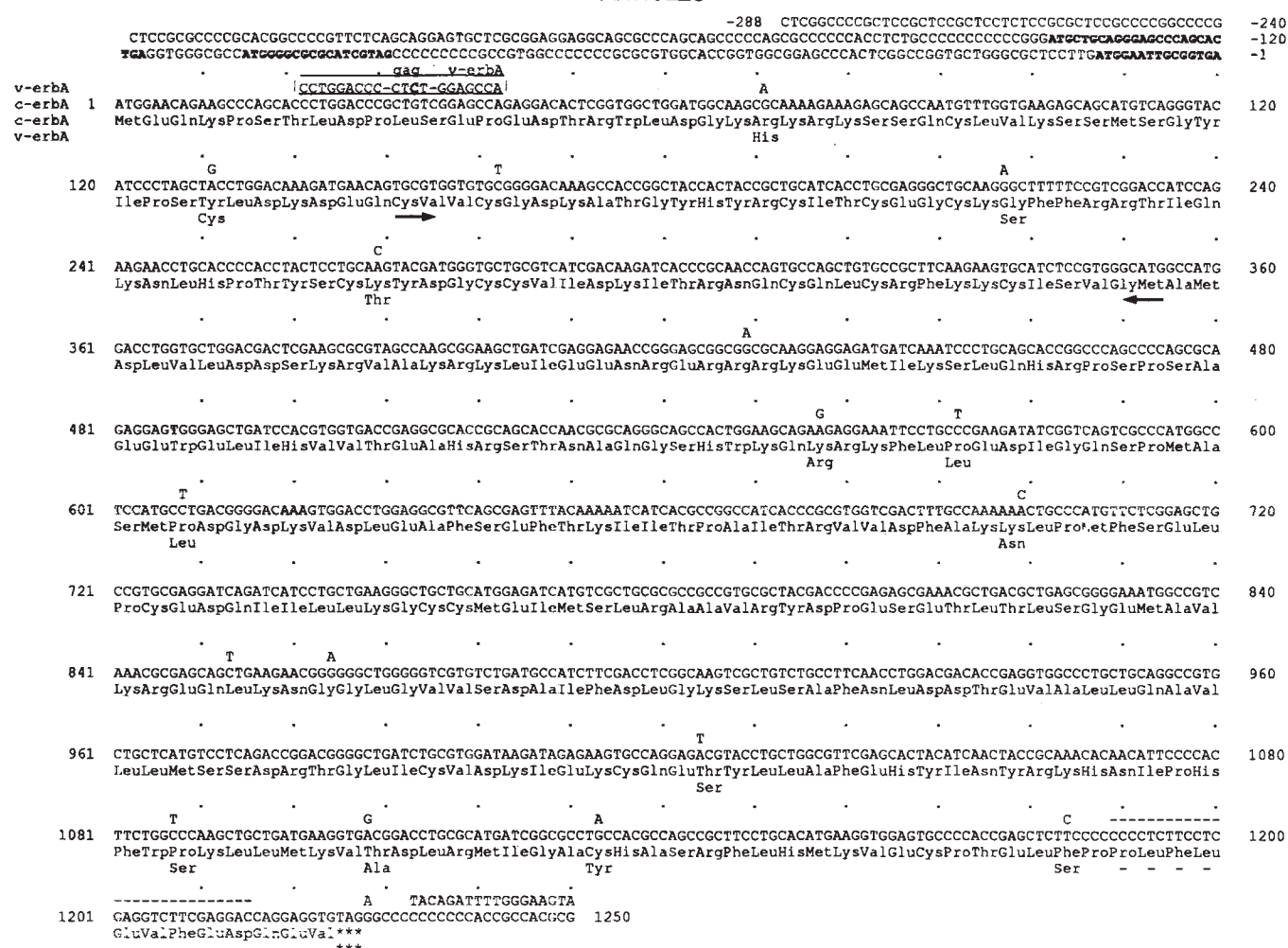


Fig. 1 Nucleotide and predicted amino-acid sequence of the *c-erb-A* cDNA clone F1, and comparison with *v-erb-A*. Only those nucleotides and amino acids in *v-erb-A* that differ from *c-erb-A* are shown, whereas deletions are indicated with bars. The three short open reading frames in the 5' untranslated region are shown in bold. The *gag* sequence homologous to *c-erb-A* and which bridges the *gag-v-erb-A* junction is boxed; nucleotide differences in this sequence are shown in bold letters and deletions with bars. The region homologous to the DNA-binding domain of steroid receptors is indicated by arrows. The U1 cDNA clone starts at nucleotide -84.

Methods. Polyadenylated RNA was isolated from a pool of 10-day-old chicken embryos⁹, and a cDNA library was constructed in λ gt11 essentially as described^{10,11}. The library ($\sim 10^6$ plaques) was screened with a nick-translated 2.5-kb *Pvu*II fragment encoding the viral *erb-A* and *erb-B* sequences from AEV. Ten *c-erb-A* positive clones were identified, which were further characterized by hybridization with probes specific for the 5' and 3' parts of *v-erb-A*. The inserts of two clones (F1 and U1) hybridizing to these probes were subsequently subcloned into the pTZ vectors¹⁴ for nucleotide sequence analysis using the dideoxy method⁵⁰. The F1 clone (1.55 kb) was sequenced in its entirety on both strands, whereas the U1 (2.1 kb) clone was subjected to partial analysis. No differences between the clones were detected in the coding or 5' non-coding sequences.

molecular mass $\sim 46,000$ (M_r 46K) for the *c-erb-A* protein. The coding sequence is preceded by a 5' untranslated region of at least 288 nucleotides, which contains three short open reading frames (shown in bold letters) 5' to the long open reading frame. In addition, a fourth reading frame, lacking an AUG codon, is open for 413 nucleotides (position -287 to 126). Further analyses are required to determine if this reading frame is used for translation by any of the *c-erb-A* mRNAs. Interestingly, the 5' untranslated sequence is very G+C rich, and a computer-assisted analysis suggested that it could form stable secondary structures (data not shown). Several of the cDNA clones isolated contained long (1–2 kb) sequences downstream of the open reading frame; their nucleotide sequences were not investigated in detail as a preliminary analysis revealed no long open reading frames, thus indicating that they represented 3' untranslated sequences. These sequences were also heterogeneous in length, suggesting that most of the cDNA clones were generated by non-specific and internal priming of the first strand cDNA synthesis.

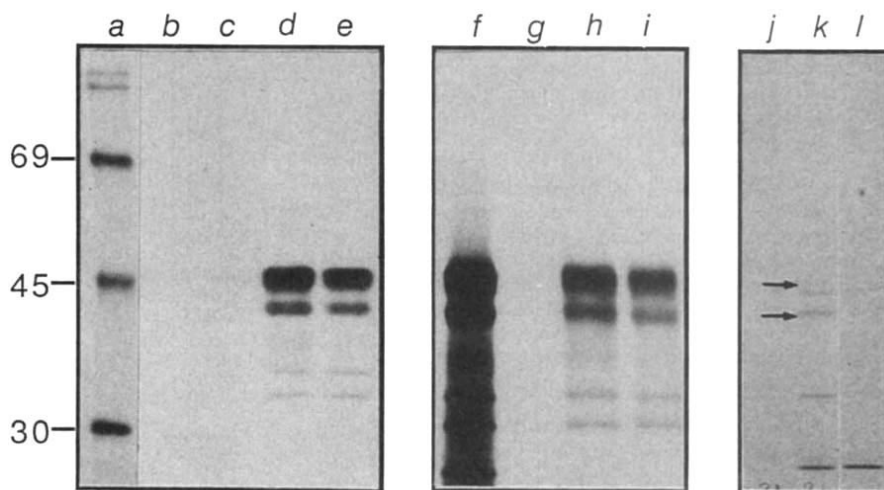
A comparison of the coding sequences of the cellular and viral^{13,12} *erb-A* genes revealed that *v-erb-A* has undergone 17

point mutations, of which two led to amino-acid substitutions located in the putative DNA-binding domain and 11 in other regions (Fig. 1). Furthermore, the *v-erb-A* protein has lost nine amino acids (nucleotides 1,188–1,215) located three amino acids before the carboxy-terminus. Finally, a comparison between the *c-erb-A* coding sequence and the *gag* sequences of Rous sarcoma virus¹³ or AEV (ref. 3) demonstrated a region with 18/22 and 19/22 nucleotide homology, respectively (boxed in Fig. 1), which bridges the junction between the *gag* and *v-erb-A* domains in p75^{gag-v-erb-A}. As a consequence, the amino-terminus of the *c-erb-A* polypeptide is 12 amino acids longer than the *erb-A*-specific part of p75^{gag-v-erb-A}, when taking into account the region homologous to *gag*. This also suggests that *c-erb-A* was fused to *gag* either by homologous recombination at the DNA level or during retrotranscription of *c-erb-A* mRNA packaged into virions.

Characterization of the *c-erb-A* protein

To determine whether the cDNA clones encode an authentic *c-erb-A* protein, one of them (clone F1) was cloned into the polylinker region of the pTZ19 plasmid vector¹⁴ to allow *in vitro*

Fig. 2 Synthesis and immunoprecipitation of the *c-erb-A* protein. Capped mRNA, synthesized *in vitro* using T7 RNA polymerase, was translated in rabbit reticulocyte lysates. Lane *a*, marker proteins (sizes shown being relative molecular masses in thousands); lane *b*, unprogrammed lysate; lane *c*, translation with RNA from clone F1 containing the 288-nucleotides long 5' untranslated sequence; lanes *d* and *e*, RNA from clones F1 and U1 with only 38 nucleotides of 5' untranslated sequence remaining. Aliquots of the translation products were immunoprecipitated with two different anti-*erb-A* antisera. Total translation products with 5'-truncated RNA (lane *f*) were immunoprecipitated with preimmune serum (lane *g*) and with two different anti-*erb-A* antisera (lanes *h* and *i*). Chicken embryo fibroblasts, transiently transfected with expression plasmids, were labelled with [³⁵S]-methionine for 2 h. Immunoprecipitations using preimmune or anti-*erb-A* antiserum with lysates from cells receiving a *c-erb-A* expression plasmid are shown in lanes *j* and *k*, respectively. Immunoprecipitation with anti-*erb-A* antiserum of lysates from cells transfected with the plasmid lacking insert is shown in lane *l*.



Methods. To reduce the length of the untranslated leader sequence, plasmids containing the entire F1 and U1 cDNA clones in the appropriate orientation in the *EcoRI* site of pTZ 19 were cleaved with *EagI* 38 nucleotides upstream of the translation initiation codon, and with *SmaI* in the polylinker region of the vector, treated with the Klenow fragment of DNA polymerase I in the presence of all four deoxynucleotide triphosphates, and religated at low DNA concentration. For *in vitro* transcription, plasmids were linearized with *HindIII* or *EcoRI*, treated with proteinase K, extracted with phenol-chloroform and precipitated. Transcription was done with T7 DNA polymerase (100 units ml⁻¹, Biolabs) at 37 °C in 40 mM Tris pH 7.9, 6 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol (DTT), 0.5 mM of the four ribonucleotide triphosphates, 0.5 mM 7mGpppG (Boehringer), 0.1 mg ml⁻¹ bovine serum albumin (BSA), 1,000 U ml⁻¹ Rnasin, at a DNA concentration of 0.1 mg ml⁻¹. Transcripts were translated in micrococcal-nuclease-treated rabbit reticulocyte lysates (NEN) in the presence of 3 mCi ml⁻¹ [³⁵S]-methionine under conditions suggested by the manufacturer. Lysates were diluted 100-fold with lysis buffer before immunoprecipitation, kept on ice for 30 min and cleared by centrifugation at 15,000 r.p.m. for 30 min. Precipitations were essentially as described^{3,51} using 5 µl of 1:10 diluted antiserum. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 10% gels, and the gels fluorographed and exposed at -70 °C. The anti-*erb-A* antisera were directed against the 5' and 3' domains of *v-erb-A* expressed in bacteria, and will be described in detail in a separate paper. To express *c-erb-A* *in vivo*, the 5'-truncated F1 clone was cloned into the expression plasmid pSV2 (ref. 15).

transcription with the T7 RNA polymerase and subsequent translation in reticulocyte lysates. In addition, to test if the 5' untranslated sequence influenced the efficiency of translation, two constructs were made with cDNA from the clones F1 and U1, each lacking 250 nucleotides of this sequence. Figure 2 shows that RNA lacking the 5' sequence (lanes *d* and *e*) directed the synthesis of a major 46K protein, whereas translation with RNA still containing this sequence gave only minor incorporation (lane *c*). Furthermore, the 46K protein as well as a minor 40K polypeptide were identified as *c-erb-A* proteins by immunoprecipitation with monospecific anti-*v-erb-A* antisera recognizing N- or C-terminal epitopes, respectively (Fig. 2, lanes *h* and *i*).

To demonstrate that the cDNAs directed the synthesis of *c-erb-A* protein also *in vivo*, chicken embryo fibroblasts were transiently transfected with a plasmid expressing *c-erb-A* from the simian virus 40 (SV40) promoter¹⁵. The cells were labelled after 48 h with [³⁵S]-methionine, lysed and subjected to immunoprecipitation with anti-*erb-A* antiserum. Figure 2 (lane *k*) shows that the *c-erb-A*-transfected cells contained 46 and 40K proteins, whereas no protein was specifically precipitated from cells transfected with the vector lacking an insert (lane *l*). The anti-*erb-A* antiserum also reacts with a 46K and a 40K protein expressed at very low levels in many types of avian cells (J.G., in preparation), suggesting that the polypeptides expressed from the cDNA represent authentic *c-erb-A* proteins.

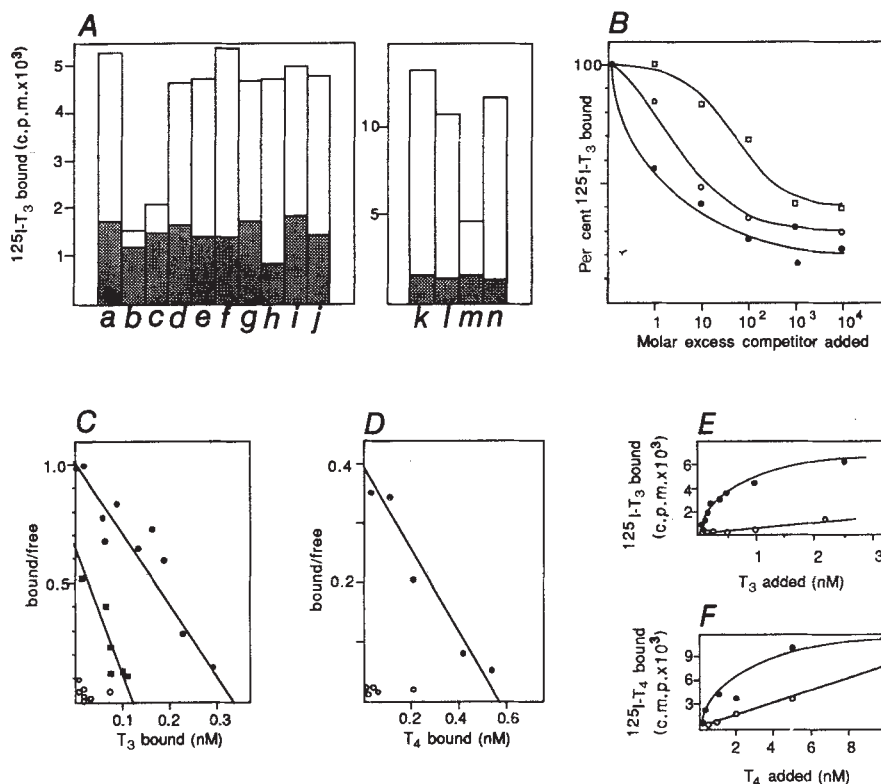
The *c-erb-A* protein binds thyroid hormone

To identify a ligand for the *c-erb-A* protein we first searched the literature for a nuclear hormone receptor expressed in many types of cells that had *M_r* ~46K. The criteria were fulfilled by the nuclear receptor for the thyroid hormone triiodothyronine (T₃), as a 47K polypeptide that binds T₃ is expressed in many

tissues^{8,16,17}. Accordingly, *c-erb-A* protein produced by *in vitro* translation in reticulocyte lysates was incubated with [¹²⁵I]-labelled T₃ in the presence or absence of a 1,000-fold excess of unlabelled hormone, and tested for binding of hormone with a filter binding assay¹⁸. Figure 3A shows that lysates programmed with *c-erb-A* RNA containing the short 5' untranslated sequence bound the radiolabelled hormone (column *a*, open), and that unlabelled T₃ (column *b*, open) or D-T₄, an analogue of T₃ which is biologically inactive but binds to the T₃ receptor¹⁹, (column *c*, open) compete with most of this binding. In contrast, the binding exhibited by mock-programmed lysates in the presence or absence of competitor (stippled columns *a*, *b* and *c*) was similar to that determined for the *c-erb-A* lysate competed with unlabelled T₃, suggesting that the *c-erb-A* protein specifically binds T₃. Similar results (data not shown) were obtained when the binding was tested by chromatography on Sephadex G75 columns²⁰. In addition, a 1,000-fold molar excess of unlabelled dexamethasone, oestradiol, progesterone, aldosterone, testosterone, vitamin D₃ (7-dehydrocholesterol), or tyrosine did not compete with radiolabelled T₃ for binding to *c-erb-A* containing lysates (open bars, Fig. 3A). Finally, an excess of these compounds did not compete with [¹²⁵I]-T₃ for nonspecific binding to mock-programmed lysates (compare the stippled columns *d-j* to *a*).

To demonstrate directly that the observed binding was due to *c-erb-A*, lysates containing *in vitro* translated *c-erb-A* protein were subjected to immunoadsorption with anti-*erb-A* or anti-*erb-B* antisera bound to protein A-Sepharose beads. The depleted samples were then assayed for binding of [¹²⁵I]-T₃ in the presence or absence of competitor. Column *m* in Fig. 3A shows that the adsorption with anti-*erb-A* antibodies removed about 70% of the specific binding activity, whereas extracts adsorbed with preimmune or anti-*erb-B* antisera exhibited no decreased

Fig. 3 Ligand-binding properties of the *c-erb-A* protein produced by *in vitro* translation. **A**, left, Reticulocyte lysates programmed with plus strand (open bars) or minus strand *c-erb-A* RNA (stippled bars) were incubated with 1 nM [125 I]- T_3 in the presence or absence of 1 μ M of various unlabelled competitors: *a*, no competitor; *b*, T_3 ; *c*, D- T_4 ; *d*, dexamethasone; *e*, oestradiol; *f*, progesterone; *g*, aldosterone; *h*, testosterone; *i*, vitamin D_3 ; *j*, tyrosine. right, open bars show that the binding activity of *c-erb-A* protein for [125 I]- T_3 was reduced in lysates treated with anti-*erb-A* but not with anti-*erb-B* or preimmune sera. Antisera: *k*, no antibodies; *l*, pre-immune serum; *m*, anti-*erb-A*; *n*, anti-*erb-B*. Stippled bars show binding in the presence of a 1,000-fold excess of unlabelled T_3 . **B**, Competition binding with analogues of T_3 . Lysates containing *c-erb-A* were incubated with 1 nM [125 I]- T_3 in the presence of the indicated molar excesses of unlabelled competitor. Reverse T_3 (rT_3) is 3,3',5'-thyronine. $\bullet$, T_3 ; $\circ$, T_4 ; $\square$, rT_3 . **C**, Scatchard analysis of T_3 binding. Lysate extracts were diluted ($\bullet$) 7-fold ($K_d = 0.33$ nM) or ($\blacksquare$) 20-fold ($K_d = 0.21$ nM); $\circ$, extract of mock-programmed lysate. **D**, Scatchard analysis of T_4 binding. $\bullet$, 7-fold diluted extract ($K_d = 1.4$ nM); $\circ$, extract of mock-programmed lysate. **E**, **F**, Saturation analysis of T_3 and T_4 binding. Binding measured in extracts of ($\bullet$) *c-erb-A* protein-containing or ($\circ$) mock-programmed lysates. Nonspecific binding was determined by parallel incubations with 1 μ M unlabelled T_3 or T_4 .



Methods. All assays were done with protein synthesized *in vitro* essentially as described in Fig. 2, except that DNA was purified on small Sepharose CL6B columns before transcription, no label was included in the translation reactions, and translation lysates were obtained from Amersham. Aliquots of the lysate were incubated overnight at 0–4 °C with L-[3',5'- 125 I]-triiodothyronine (3,000 mCi mg⁻¹) or L-[3',5'- 125 I]-thyroxine (1,500 mCi mg⁻¹, both Amersham) at the indicated concentrations (generally 0.5–2.0 μ l lysate in a total volume of 10 μ l) in binding buffer (50 mM NaCl, 2 mM EDTA, 5 mM mercaptoethanol, 20 mM Tris pH 7.5, 10% glycerol). Radiolabelled hormone was dried under vacuum to remove ethanol when the final isotope concentration used would exceed 2 nM. Bound radiolabelled hormone was determined¹⁸ by filtration at 4 °C using Millipore HAWP 02500 filters pretreated with 3.5 ml binding buffer. Filters were washed by suction with 5 ml binding buffer at 4 °C three times, and were counted in a gamma counter. Control lysates were programmed with RNA transcribed from a clone with the *c-erb-A* cDNA in the opposite orientation. For immunodepletion, 5 μ l of programmed lysate was diluted fivefold with binding buffer, incubated with 2 μ l undiluted antiserum for 4 h at 4 °C, and adsorbed for 30 min to protein-A-Sepharose (saturated with erythroblast extract and washed extensively with binding buffer).

binding (columns *l* and *n*). Finally, the proteins adsorbed to the anti-*erb-A* antibodies bound no T_3 , presumably due to conformational changes or steric hindrance imposed by the antibodies (data not shown).

The receptors for thyroid hormone bind with lower affinity analogues^{21,22} of T_3 , such as thyroxine (T_4) and reverse T_3 (rT_3). We therefore tested if higher concentrations of these analogues would be required to compete with labelled T_3 for binding to *c-erb-A* protein. Figure 3B shows that T_4 was 5–10-fold and rT_3 was 50–100-fold less efficient than unlabelled T_3 in competing with [125 I]- T_3 . These relative efficiencies are similar to those reported for competition of [125 I]- T_3 binding to the thyroid hormone receptors^{21,22} and reflect the different biological activities of these products.

To determine accurately the affinity of *c-erb-A* protein for thyroid hormone, we performed Scatchard analyses²³ with labelled T_3 and T_4 . Because these hormones have been reported to bind with low affinity to unspecific sites at high protein concentrations²⁴ which affect the dissociation constants measured, we determined the K_d of *c-erb-A* for T_3 using reticulocyte lysates diluted to different concentrations. Figure 3C (filled symbols) shows that the K_d for T_3 was between 0.21 and 0.33 nM with 20- and 7-fold diluted extracts, respectively, whereas the K_d for T_4 , determined in a separate experiment with a 7-fold diluted extract, was 1.4 nM (Fig. 3D). In contrast, Scatchard analyses done with mock-programmed lysates only indicated the presence of low-affinity/high-capacity binding sites (open symbols). The

affinities of the *c-erb-A* protein for T_3 and T_4 are similar to the published values obtained with the thyroid hormone receptor in whole cells and in crude nuclear extracts^{22,25}.

Finally, the results from the saturation binding experiments (Fig. 3E and F) showed that the high-affinity sites (closed symbols) were saturable with low concentrations of hormone, whereas the low-affinity sites present in the mock-programmed lysates were non-saturable (open symbols). By calculating the amount of [35 S]-methionine-labelled *c-erb-A* protein in the lysates and comparing to the amount of T_3 bound, we estimate that 5–10% of the total *in vitro* synthesized protein bound radiolabelled hormone, a result similar to those found for *in vitro* synthesized steroid receptors²⁶.

Cellular but not viral *erb-A* protein binds T_3

The T_3 receptor is nuclear regardless of whether or not it has bound hormone⁸. To verify that the *c-erb-A* protein binds T_3 also *in vivo*, and to test the hormone binding capacity of p75^{gag-v-erb-A}, chicken embryo fibroblasts containing *c-erb-A* or *v-erb-A* were tested for hormone binding. The *c-erb-A* gene was expressed from a replication-competent retrovirus vector²⁷, whereas a *wt* and an inactive *v-erb-A* gene (*v-erb-A2*⁻, ref. 1) were expressed from retrovirus constructs also conferring resistance to the drug G418. To measure nuclear T_3 binding *in vivo*, transfected and fully infected cell cultures were incubated with [125 I]- T_3 for three hours, and the radioactivity in the isolated nuclei determined. Figure 4 (left) shows that the nuclei (open

Fig. 4 Hormone-binding properties of *c-erb-A* and *v-erb-A* proteins produced *in vivo*. Left panel, Cells expressing *c-* or *v-erb-A* were incubated for 3 h with [125 I]- T_3 , and the radioactivity in the nuclei determined. Middle, Hormone binding by nuclear extracts incubated with [125 I]- T_3 *in vitro*. Right panel, Reduction of *in vitro* binding activity in nuclear extracts by adsorption with anti-*erb-A* antisera. Open bars show binding of radiolabelled hormone in the absence of competitor, whereas stippled bars indicate binding of [125 I]- T_3 in the presence of a 1,000-fold excess of unlabelled hormone.

Methods. The F1 cDNA clone containing only the short 5' leader sequence was cloned into a modification of the replication competent avian retrovirus vector 779/989, a derivative of a previously described vector²⁷. Virus constructs expressing *wt* or mutant (*v-erb-A2*⁻, ref. 1) *v-erb-A* genes were constructed by excising the *v-erb-B* gene from AEV with *Apa*I and *Eco*RI, followed by appropriate modification and insertion of the *Bgl*III–*Bam*HI fragment of the Tn5 *neo*^R element. The constructs were introduced into chicken embryo fibroblast cells by the $Ca_3(PO_4)_2$ precipitation technique^{52,53}. In the case of the *c-erb-A* construct, virus infection was monitored by reverse transcriptase activity in the culture media, whereas cells containing the *v-erb-A-neo*^R viruses were superinfected with RAV1 helper virus and then isolated after selection for G418 resistance. Control experiments showed that these viruses expressed *v-erb-A* at levels similar to those found in AEV-transformed cells (not shown). Cells were grown in Eagle's medium plus 5% fetal calf serum and 2% heat-inactivated chicken serum. For analysis of nuclear T_3 -receptor content, cells grown for 24–48 h in media containing T_3 -depleted⁵⁴ serum were trypsinized, washed twice with Hank's and resuspended at $\sim 3 \times 10^6$ cells ml⁻¹ in T_3 -free medium supplemented with 0.15 nM [125 I]- T_3 for 3 h. Cells were subsequently washed with Hank's, and lysed in 1 ml 50 mM Tris pH 7.85, 1.1 mM MgCl₂, 0.5% Triton-X-100 at 0°C. Nuclei were collected by centrifugation (1,500 r.p.m. for 5 min) and were washed once more in lysis buffer before counting. GH₁ cells were cultured in Eagle's medium containing 10% horse serum and 2.5% fetal calf serum. Isolation of nuclei and preparation of nuclear extracts were done employing techniques used for isolation of nuclear T_3 receptor⁵⁵, except that inhibitors of proteolysis were included in all steps of isolation. Experiments on [125 I]- T_3 binding with nuclear extracts and immunodepletion were done as described in Fig. 3.

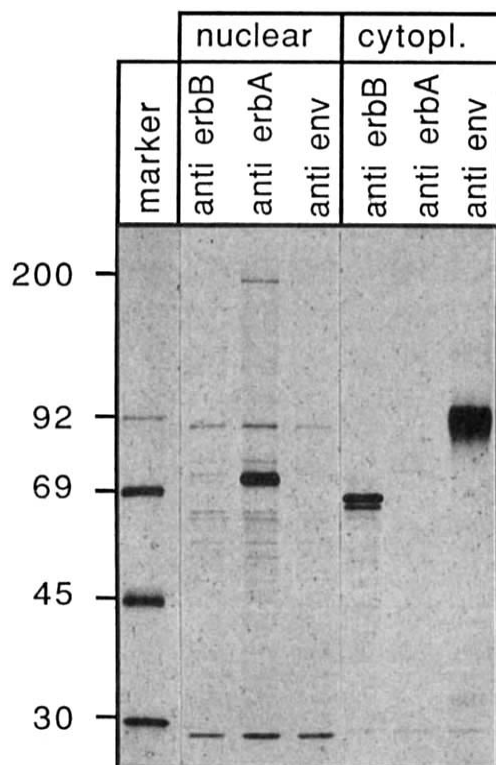
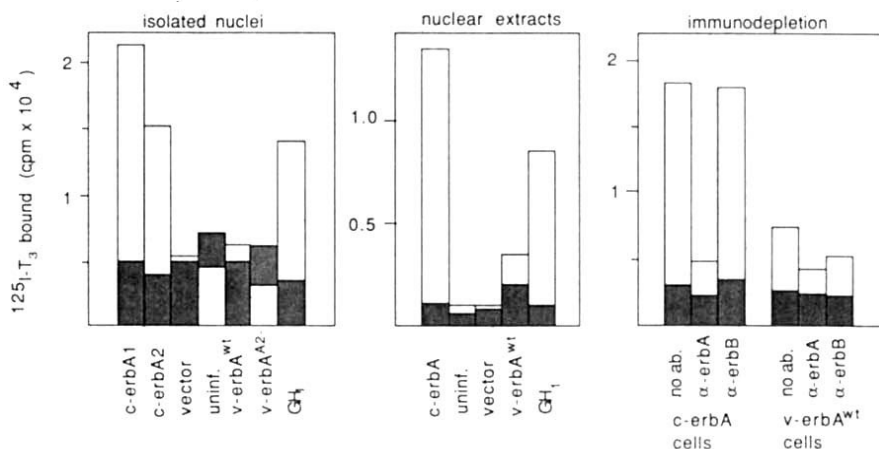


Fig. 5 Demonstration of a nuclear localization for the *v-erb-A* protein. Cell fractionation experiments with AEV-transformed erythroblasts were done as described in Fig. 4. Labelling of cells and immunoprecipitations were carried out as described in Fig. 2. Sizes for markers are relative molecular masses in thousands.

columns) from two different cultures of *c-erb-A* expressing cells (*c-erb-A*1 and 2) bound [125 I]- T_3 at levels similar to that found in the nuclei of GH₁ cells, a rat pituitary line expressing $1-2 \times 10^4$ T_3 receptors per cell^{28,29}. In addition, a 1,000-fold excess of unlabelled T_3 competed with this binding (stippled columns). In contrast, only a low amount of specific T_3 binding was found in the nuclei from the *wt* and the mutant *v-erb-A* expressing cells, or the cells infected with the replication competent vector lacking *c-erb-A* insert. Similar results were obtained when nuclear extracts, prepared from unlabelled cells, were tested for binding of [125 I]- T_3 *in vitro* (middle panel). Again, receptors from the *c-erb-A* and the control GH₁ cells bound radioactivity at levels 8–12-fold higher (open bars) than that observed when binding was done in the presence of an excess of unlabelled T_3 (stippled bars). In contrast, the binding seen with extracts from uninfected cells or cells expressing *v-erb-A* was only 1.3–1.7 times background. Lastly, adsorption with anti-*erb-A* antisera of nuclear extracts from *c-erb-A* but not *v-erb-A* cells reduced the specific binding by 77% (Fig. 4, right panel), demonstrating that the high T_3 binding was dependent on *c-erb-A* protein.

To exclude the possibility that lack of nuclear hormone binding by *v-erb-A*-expressing cells was due to cytoplasmic localization of p75^{gag-v-erb-A}, a cell fractionation experiment was done. Erythroblasts transformed by AEV were labelled with [35 S]-methionine, and nuclear and cytoplasmic extracts, prepared using techniques described for the isolation of the T_3 receptor¹⁷, were immunoprecipitated with antisera against *v-erb-A*, *v-erb-B* and the viral envelope glycoprotein. Figure 5 shows that the *v-erb-A* protein was present predominantly in the nuclear extract, whereas the *v-erb-B* and *env* proteins partitioned in the cytoplasmic fraction. Previous reports have suggested that *v-erb-A* is localized in the cytosol³⁰; these cell fractionation experiments were however done under conditions that may not preserve the nuclear localization of at least the unoccupied oestradial receptor^{31,32}.

Discussion

Several lines of evidence suggest that the avian *c-erb-A* gene codes for a thyroid hormone receptor: the molecular weight of the *c-erb-A* protein is the same as that of a previously characterized receptor; it binds T_3 and its analogues with affinities typical of the T_3 receptor; binding activity can be removed with anti-*erb-A* antibodies; and both the thyroid hormone receptor and *c-erb-A* are expressed at low levels in many tissues. Finally, Weinberger and collaborators have demonstrated that a human *c-erb-A* protein also binds thyroid hormone *in vitro*³³. However, the evidence is so far based solely on hormone-binding experiments, and definite proof will require the demonstration that the *c-erb-A* protein exerts the same effects *in vivo* as the thyroid hormone receptor.

Thyroid hormone induces a wide variety of metabolic effects, for instance increased lipogenesis and ketogenesis. These effects have been studied in great detail in liver cells but have also been observed in many other types of tissues⁸. The hormone also plays key roles in differentiation, such as the rapid development of frogs from thyrectomized tadpoles, the induction of growth hormone production in the pituitary, and the development of the brain in neonates by promoting dendrite formation and myelination⁸. Interestingly, T_3 does not induce the same physiological effects in the brain as in other tissues³⁴, suggesting that the brain receptor could be distinct from the receptor(s) found in other cells. In fact, several *c-erb-A* related genes appear to exist in the human genome. A *c-erb-A* gene highly homologous to the avian gene reported in this paper is located on human chromosome 17 (refs 35–38), whereas other distinct genes have been found on chromosome 3 (ref. 33).

The thyroid hormone receptor also has a nuclear localization in the unoccupied state as well, and is thought to act by binding to DNA (refs 39, 40) thus inducing or suppressing gene expression in a manner similar to that of steroid receptors⁴¹. The *v-erb-A* protein binds no hormone, yet it is nuclear, binds to DNA *in vitro* (H.B. and U. Gehring, unpublished data) and exerts specific physiological effects in erythroblasts³. It is therefore likely that p75^{gag-v-erb-A} has lost its hormone-binding capacity as a result of one or several of the mutations it has accumulated. Moreover, it is unclear whether it affects the expression of the same genes and in the same manner as the normal receptor,

as its presumed DNA-binding region has two amino-acid substitutions. It is possible that p75^{gag-v-erb-A}, although unable to bind ligand, acts in a similar fashion as the increased nuclear transfer (ntⁱ) mutants previously described for the glucocorticoid receptor⁴¹.

The maturation of erythroid cells is accompanied by synthesis of several components important for proper red cell differentiation, such as band 3, the major anion transporter which also serves as an anchor for erythrocyte cytoskeletal proteins, and hemin, a possible regulatory factor of differentiation^{42,43}. Interestingly, thyroid hormone regulates the induction of key liver enzymes involved in hemin synthesis⁴⁴, and induces increased levels of the Na^+/K^+ ATPase^{45,46}. We are therefore currently investigating the possibility that *v-erb-A* exerts its effects in erythroblasts by suppressing or inducing the synthesis of ion transporters and hemin.

It has previously been demonstrated that thyroid status has a modulating effect on neoplasia. Administration of thyroid hormone to thyrectomized rodents is a prerequisite for the induction of hepatomas by certain chemicals, indicating a role in the initiating action of the carcinogen. In contrast, thyroid hormone appears to promote the growth and metastatic potential of other types of neoplasias, demonstrating an enhancement of tumour progression⁴⁷. In addition, the transformation of cultured cells by radiation or adenovirus infection is facilitated by thyroid hormone⁴⁸. The *v-erb-A* oncogene of AEV enhances the transforming activity of other oncogenes in erythroblasts but also in fibroblasts (refs 1–3, and M. Jansson and B.V., unpublished data). In addition, the integration of a hepatitis B viral genome close to a human *erb-A* related gene was recently reported⁴⁹. The demonstration that the *c-erb-A* protein has properties similar to those of the T_3 receptor may thus represent the first identification of an oncogene that enhances oncogenesis without being able to induce detectable neoplastic effects on its own.

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The *c-erb-A* gene encodes a thyroid hormone receptor

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The cDNA sequence of human c-erb-A, the cellular counterpart of the viral oncogene v-erb-A, indicates that the protein encoded by the gene is related to the steroid hormone receptors. Binding studies with the protein show it to be a receptor for thyroid hormones.

THE interaction of DNA-binding proteins with specific DNA sequences is one mechanism by which gene transcription is controlled. Characterization of the regulatory proteins, which include steroid hormone receptors, should help our understanding of DNA-protein interactions and of the control of gene activation and repression. The human glucocorticoid receptor (hGR) complementary DNA has been sequenced^{1,2} and shown to be functionally active³. Interestingly, sequence analysis of the receptor showed it to be related to the product of the *v-erb-A* oncogene product of avian erythroblastosis virus (AEV)⁴. This led to the proposal that the steroid receptors and the *erb-A* oncogene products share a common primordial archetype and that the *erb-A* proto-oncogene products may also be proteins that bind to DNA enhancer elements. Recent characterization of the human oestrogen^{5,6}, chicken progesterone^{7,8} and human aldosterone (J. Arriza, C. W. and R.M.E., unpublished data) receptors further support these conclusions.

Accordingly, we started characterizing the human *c-erb-A* proto-oncogene even though its functional identification could not be ensured. During the progress of these studies, advances were made in detailing the functional domains of the glucocorticoid receptor³ which indicated that the hGR hormone binding domain was unusually large, encompassing the carboxy-terminal 300 amino acids. This entire region has distant but significant similarity to the carboxy terminus of *v-erb-A* which therefore focused our attention on classes of molecules that might exert transcriptional regulatory effects similar to those of steroid hormones.

The molecular mechanism of thyroid hormone stimulation of gene expression may be similar to that outlined for steroids⁹. Thyroid hormone is present in all chordate species examined and exerts profound effects on development and differentiation, such as metamorphosis in amphibians⁹. Like steroids, thyroid hormones may enter cells by passive diffusion and bind to high affinity nuclear receptors which in turn mediate a rapid and selective activation of gene expression^{10,11}. Evidence favouring this hypothesis has come largely from studies of the induction of growth hormone and its messenger RNA in the somatotroph of the rat anterior pituitary and in a number of related rat somatotrophic cell lines^{12,13}. Thyroid hormones rapidly increase the transcription of the growth hormone gene in these cells^{14,15}. The increase in transcription is accompanied by increased levels of nuclear thyroid hormone-receptor complexes, is time- and concentration-dependent, and is independent of protein synthesis¹⁶⁻¹⁸.

The similarity of steroid and thyroid hormone actions led us to examine the possibility that the *erb-A* protein may itself be the thyroid hormone receptor. We first isolated and characterized a cDNA for human *c-erb-A*. The sequence predicts a 456 amino-acid polypeptide containing a cysteine/lysine/arginine-

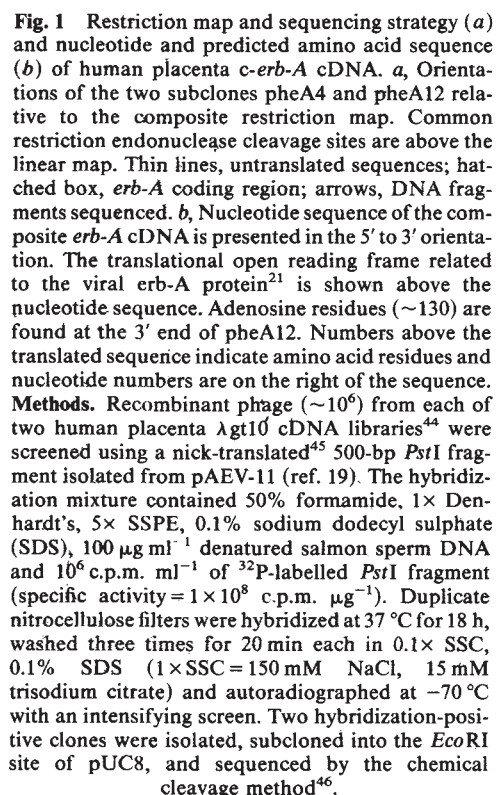
rich region similar to the putative DNA-binding domain of steroid hormone receptors and a carboxy-terminal region distantly related to the steroid binding domain. Using a functional assay developed for analysing the hormone binding properties of cloned steroid receptors², we demonstrated that the translation product from the human *c-erb-A* cDNA possesses intrinsic thyroid hormone binding activity, characteristic of the native thyroid hormone receptor molecule.

Characterization of *c-erb-A* cDNAs

To isolate a human *c-erb-A* cDNA, a 500-base pair (bp) *Pst*I DNA fragment isolated from a region of the AEV genome containing only the *v-erb-A* gene¹⁹ was used as a ³²P-labelled probe to screen two human placenta cDNA libraries. Two overlapping λ gt10 cDNA clones were obtained by screening two independent libraries of $\sim 10^6$ phage recombinants each. The restriction maps were deduced for each of the cDNA clone *Eco*RI inserts from pUC8 subclones, pheA4 and pheA12 (Fig. 1a). Nucleotide sequence analysis of these overlapping 1.5 kilobase (kb) cDNA clones revealed that the composite sequence contains a long open reading frame of 456 amino acids with a presumptive initiator methionine codon at nucleotide number 301 and a terminator codon at 1,669 in the sequence (Fig. 1b). Seven codons upstream of the ATG is an in-frame terminator TAA providing support for the initiator methionine, although another methionine found 26 codons downstream makes this assignment tentative. No consensus polyadenylation addition signal (AATAAA, ref. 20) is discernible in the 27 nucleotides between the terminator and poly(A) tract in pheA12.

A predicted polypeptide of relative molecular mass 52,000 ($M_r = 52K$), encoded within the human *c-erb-A* cDNA translational open reading frame, shares 82% amino acid identity with the region downstream from the viral *gag* sequences²¹ in AEV. No gaps in the amino acid comparison were revealed. The human *c-erb-A* amino acid sequence is homologous with the viral protein beginning at viral amino acid residue 37 (Fig. 2). The carboxy-terminus of *c-erb-A* differs from that of *v-erb-A*: amino acid sequence similarity of the two polypeptides terminates at residue 445 of *c-erb-A* and residue 380 of *v-erb-A* (Fig. 2).

Alignment of the human *c-erb-A* nucleic acid sequence with that of *v-erb-A* shows that $\sim 74\%$ of the human gene is identical to the viral gene in the region of amino acid homology (nucleotides 563-1636 in *c-erb-A*, data not shown). These comparisons, coupled with previous data describing other human *erb-A* genes mapping to chromosome 17 (refs 22-24), indicate that the human placenta *c-erb-A* gene we have isolated is distinct. One of the two chromosome 17 *erb-A* genes (both of which we propose to call *hc-erb-A α*) has 82% similarity with the *v-erb-A* gene by nucleotide sequence and 89% by amino acid identity²⁴. There-



Hybridization of restriction endonuclease-digested human placenta DNA with a labelled DNA fragment derived from the cysteine-rich region of the c-*erb-A* polypeptide (Fig. 1*a,b*) pro-

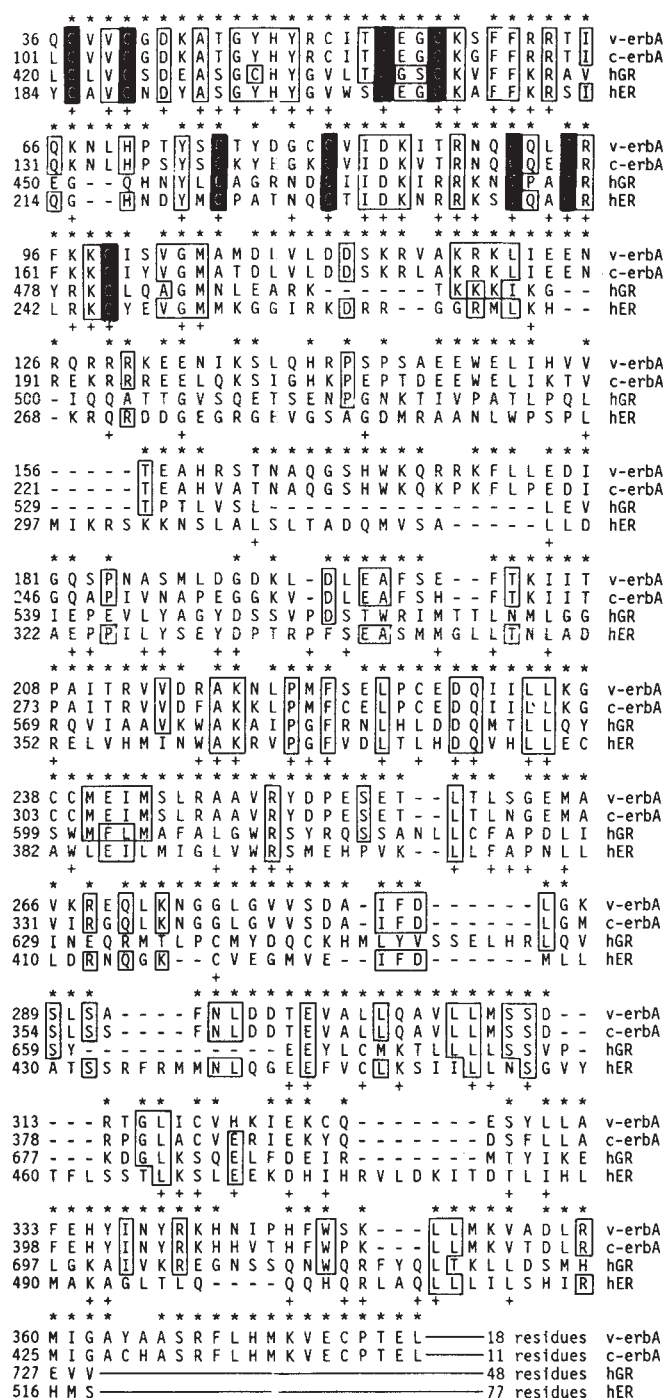


Fig. 2 Amino acid sequence comparison between the carboxy-terminal portions of the *v-erbA* oncogene product, the human placenta *c-erbA* polypeptide and the human glucocorticoid and oestrogen receptors. Translated amino acid sequences for both the *v-erbA* protein (upper sequence) and the human placenta *c-erbA* polypeptide (second sequence) were compared by aligning matching residues. A computer program for the concurrent comparison of three or more amino acid sequences⁴⁷ was used to align *c-erbA*, human glucocorticoid receptor (hGR third sequence from top)² and human oestrogen receptor (hER, bottom sequence)⁵ carboxy-terminal amino acid sequences, on the basis of progressive evaluation of selected segments from each sequence. Amino acid residues matched in at least three of the polypeptides are boxed. Amino acid matches between the two *erbA* polypeptides are indicated by an asterisk above the top sequence in each column. Amino acid identities between the steroid receptors are designated by crosses below the sequences. Hyphens, gaps inserted to maximize the no. of matches in the comparison. Cysteine residues conserved between the four polypeptides printed white-on-black.

duced two bands in every digestion with the exception of *PvuII* (Fig. 3a). The greater intensity of the 9.4 kb-band suggested that it contains two hybridizing DNA fragments. When the hybridization conditions were relaxed, additional bands were observed in the products of each enzyme digestion. For example, two faint bands of 5.1 and 3.6 kb were seen after *PstI* digestion (Fig. 3b). The hybridization probe contains a single internal *PstI* site (Fig. 1a) which probably explains the increased number of *PstI* bands detected with this probe.

Similar high-stringency hybridization experiments were performed using a 260 bp *EcoRI*-*BamHI* fragment from the 5' untranslated region of *pheA4* (Fig. 1a and data not shown). Two hybridizing DNA fragments were detected with all restriction enzymes providing further support for the existence of two related *c-erbA* genes. No additional DNA bands were seen when the hybridization was performed under relaxed conditions using this probe (data not shown). We conclude that there are two closely related *hc-erbA* proto-oncogenes and a third, less similar one, in the human genome.

Laser-sorted chromosomes were prepared from human lymphoid cells²⁵, bound to nitrocellulose filters and hybridized under non-stringent hybridization and washing conditions using the 1.5-kb *EcoRI* insert from *pheA4* (Fig. 3c). This probe detected only human chromosome 3-specific DNA and suggests that the three *c-erbA*-related genes are chromosomally linked, although we cannot strictly exclude the possibility that the non-stringently hybridizing *erbA* gene maps in another chromosome. Interestingly, another steroid receptor/*erbA* genomic fragment has recently been identified by characterizing the integration site for hepatitis B virus in a human hepatocellular carcinoma²⁶. This locus has also been mapped on human chromosome 3 suggesting that the *erbA* genes may be closely linked.

Expression of *c-erbA* genes

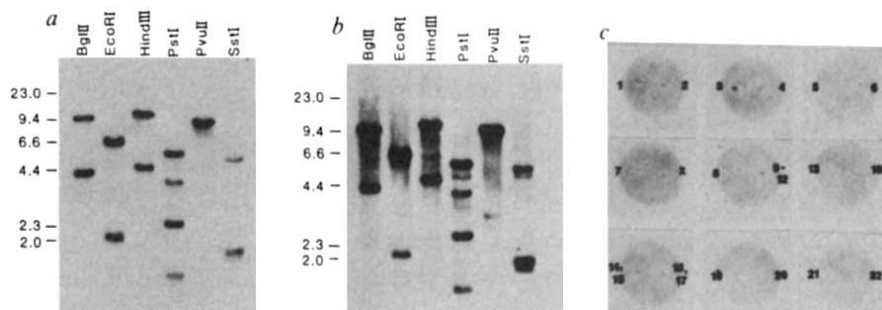
Northern blots hybridizing cytoplasmic poly(A)-containing RNAs, isolated from various human cell lines or human term placenta, with a 650-bp *BamHI*-*PstI* fragment from *pheA4* (Fig. 1a) revealed a single RNA species of 2,000 nucleotides that is most abundant in HeLa and MCF-7 cells (Fig. 4a). The size of the mRNA indicates that we have isolated a nearly full-length *c-erbA* cDNA. HT1080 cells contain a small amount of the 2-kb transcript while it is undetectable in IM-9 cells. Human placenta appears to contain multiple species of 5, 3 and 2.5 as well as 2.0 kb RNA. It is unclear whether the multiple placenta bands represent nuclear precursors, or mature mRNAs from a single gene, or the products of other *erbA* genes.

The protein products of the human *c-erbA* cDNA were characterized by *in vitro* translation. A cDNA containing the entire *c-erbA* coding region was inserted into the *EcoRI* site of the expression vector pGEM3 in both orientations. Capped RNA transcripts synthesized by T7 polymerase from these templates were used to programme protein synthesis in rabbit reticulocyte lysates and the ³⁵S-methionine-labelled products were separated on SDS-polyacrylamide gels²⁷. Proteins with *M_r* of 55, 52, and 35K were detected when *peA101* DNA (*erbA* sense transcripts) was used as template (Fig. 4b, lanes 3 and 4) but not when *peA102* DNA (*erbA* antisense transcripts) was used (Fig. 4b, lane 2). The 55 and 52K products may correspond to polypeptides initiating translation at methionines 1 and 27, respectively (Fig. 1), while the 35K product may be a proteolytic breakdown product.

Thyroid hormone binding

Structural similarity of the steroid ligands as well as the partial amino acid sequence homology (40%) between the carboxy termini of the hGR and hER (which specify the hormone binding domains, ref. 28) support the hypothesis that the steroid receptors comprise a family of regulatory proteins. The more distant

Fig. 3 Southern analysis and chromosome mapping of human placenta DNA with *c-erb-A* DNA probes. *a*, Human term placenta DNA was digested with restriction endonucleases and products were separated on a 0.8% agarose gel. DNAs were transferred to nitrocellulose paper⁴⁸ and hybridized in 50% formamide, 5× SSPE, 1× Denhardt's, 0.1% SDS, 100 µg ml⁻¹ salmon sperm DNA with the 450-bp *Sst*I fragment from *pheA4* which was nick-translated to a specific activity of 5×10⁸ c.p.m. µg⁻¹. The filter was washed in 0.1× SSC, 0.1% SDS at 60 °C and exposed to X-ray film at -70 °C with an intensifying screen. Lambda *Hind*III DNA markers (size in kb) are aligned to the left of the autoradiogram. *b*, Analysis of placenta DNA using the same *c-erb-A* probe as in *a* under non-stringent hybridization conditions. A parallel blot containing the same samples was hybridized as in *a*, except that 35% formamide was used. The hybridized filter was washed in 2× SSC, 0.1% SDS at 55 °C and exposed to X-ray film as described above. *c*, Chromosome mapping of human *c-erb-A* genes. Human lymphocyte chromosomes were separated by laser cytofluorometry²⁵ and probed using the non-stringent hybridization conditions described in *b*, but with the 1.5 kbp *Eco*RI insert from *pheA4* as probe.



homology between the carboxy terminus of *c-erb-A* and the steroid receptors suggested that *erb-A* proto-oncogenes probably do not encode steroid receptors, but is consistent with the hypothesis that *erb-A* may respond to other molecules. We have shown that *in vitro* translation can be used as a means to characterize hormone binding activity of cloned steroid receptors². Accordingly, this assay was employed to identify the putative *c-erb-A* ligand.

Steroid and thyroid hormones exert their effects through fundamentally similar mechanisms. To address the possibility that *erb-A* may be the thyroid hormone receptor, the *in vitro* translation products were mixed with ¹²⁵I-3,5,3'-triiodo-L-thyronine (¹²⁵I-T₃) in a hormone binding reaction²⁹. Non-specific hormone binding was determined by adding a 500-fold molar excess of cold T₃ to parallel samples. Remarkably, the mixture containing the 55 and 52K polypeptides (peA101) acquired ¹²⁵I-T₃ binding whereas the anti-sense RNA-programmed lysates (peA102) had only background binding. Hormone binding was sensitive to proteases but not nucleases (data not shown). Affinity of T₃ for the cloned *erb-A* protein was determined by Scatchard analysis (Fig. 5a). A K_d value of 5×10⁻¹¹ M was obtained, which is virtually identical to the T₃ binding (6×10⁻¹¹ M) in HeLa cell nuclear extracts (data not shown).

Specific analogues of thyroid hormones have characteristic competition patterns for T₃ binding to the native thyroid hormone receptor. We determined whether the *erb-A* product synthesized *in vitro* shared the same intrinsic hierarchy of affinities for a range of natural and synthetic thyroid hormones. Competition of ¹²⁵I-T₃ binding was most effectively achieved with 3,5', 3'-triiodothyroacetic acid (TRIAc) which inhibited 50% of ¹²⁵I-T₃ binding at 300 pM (Fig. 5b). In addition, L-thyroxine (Fig. 5b), D-T₃ and reverse T₃ (3,3',5'-triiodo-L-thyronine) competed more poorly than T₃ (Fig. 5c), while 100 µM vitamin D₃, aldosterone, cortisol, testosterone, progesterone or oestradiol did not compete (data not shown), consistent with the biochemical properties of the rat thyroid hormone receptor²⁹⁻³¹.

High salt (0.4 M KCl) HeLa cell nuclear extracts contained thyroid hormone (¹²⁵I-T₃) binding activity, while none was found in cytoplasmic or low-salt (0.1 M KCl) nuclear extracts (data not shown). Competition of thyroid hormone binding in the nuclear extracts was quantitatively similar to that of lysates containing *c-erb-A* made *in vitro* (compare Fig. 5b and d). Furthermore, thyroid hormone binding using 0.4 M KCl nuclear extracts from IM-9 cells, which contain undetectable levels of *c-erb-A* mRNA (Fig. 4, lane 4), is negligible when compared with similar HeLa extracts (data not shown). These results provide direct evidence that *c-erb-A* is the thyroid hormone receptor.

Conclusions

The data in this report provide three criteria that identify the *c-erb-A* product as the thyroid hormone receptor. First, the overall structural homology of *c-erb-A* to steroid hormone receptors suggests that *erb-A* is likely to be a ligand-responsive regulatory protein. Second, the expressed protein product has the same intrinsic hierarchy of affinities for natural and synthetic thyroid hormones as the native receptor. Third, the molecular weights of *erb-A* *in vitro* translation products are similar to the photo-affinity-labelled rat thyroid hormone receptor³⁹. The identity of *erb-A* as the thyroid hormone receptor could be further sub-

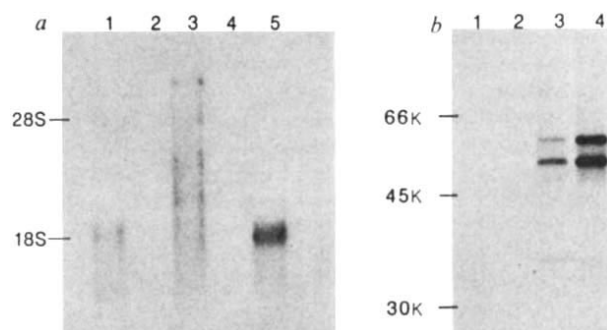
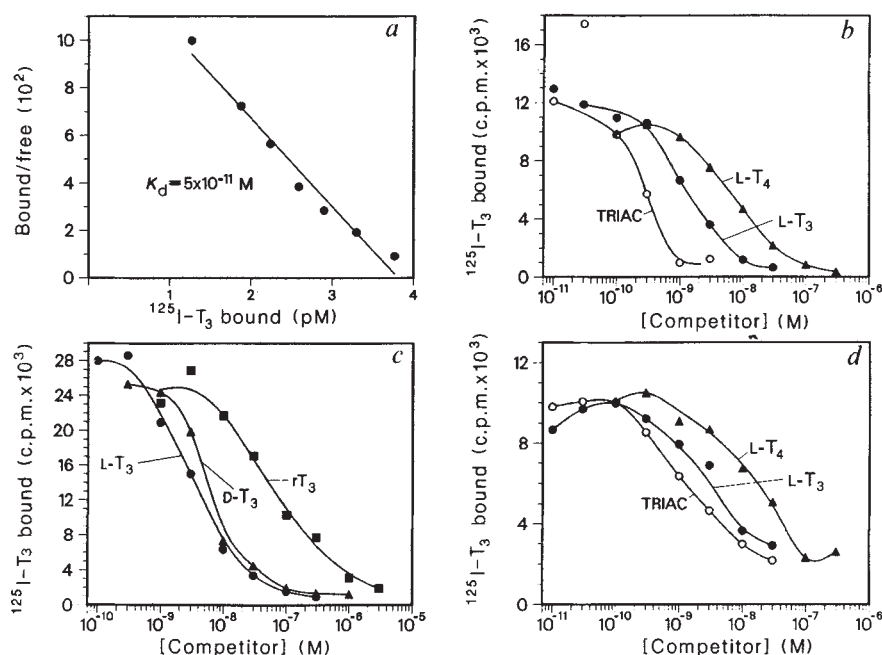


Fig. 4 Human *c-erb-A* expression. *a*, Northern analysis of RNAs from human cell lines and human placenta. Cytoplasmic poly(A)-containing RNA (12 µg) from HeLa, MCF-7 and IM-9 cells, or total poly(A) RNAs from HT1080 or placenta, were separated on a 1% agarose gel containing formaldehyde, transferred to nitrocellulose⁴⁹ and probed using a nick-translated 650-bp *Bam*HI-*Pst*I *pheA4* fragment. Cytoplasmic RNAs were isolated from the cell lines using isotonic buffer and 0.5% NP40, while the placenta RNAs were extracted from fresh tissue using guanidine thiocyanate⁵⁰. Lane 1, HeLa; lane 2, HT1080; lane 3, human placenta; lane 4, IM-9; lane 5, MCF-7. *b*, Synthesis of *erb-A* polypeptides *in vitro*. The ³⁵S-methionine-labelled products synthesized using T7 polymerase-catalysed RNA transcripts were separated on a 12.5% SDS-polyacrylamide gel which was fluorographed (EN³HANCE, NEN). Lane 1, control (without mRNA); lane 2, peA102 (anti-sense RNA, 4 µl); lane 3, peA101 (sense RNA, 1 µl); lane 4, peA101, (4 µl RNA). Sizes of protein standards: bovine serum albumin, 66.2K; ovalbumin, 45K and carbonic anhydrase, 31K. **Methods.** The *Eco*RI insert from *pheA12* which contains the entire coding region of *c-erb-A* was inserted into the *Eco*RI site of pGEM3 (Promega Biotec) in both orientations. Plasmid DNAs peA101 and peA102 were linearized with *Hind*III, purified on 0.8% agarose gels and used as templates for T7 polymerase-catalysed synthesis of RNAs *in vitro*². After P60 chromatography, total nucleic acid material (2 µg) was used to programme protein translation in a rabbit reticulocyte lysate system (Promega Biotec) with ³⁵S-methionine (25 µCi, 1100 Ci mmol⁻¹, NEN) final volume 25 µl.

Fig. 5 Thyroid hormone binding to erb-A polypeptides synthesized *in vitro*. *a*, Scatchard analysis of ^{125}I -T₃ binding to the erb-A polypeptides made *in vitro*. The erb-A polypeptides (2 μl from the *in vitro* translation mixture in a total volume of 2 ml) were assayed for specific thyroid hormone binding activity using hydroxylapatite to measure the amount of bound and free labelled hormone at different concentrations of ^{125}I -T₃ as described⁵¹. *b*, Competition of thyroid hormone analogues for ^{125}I -T₃ binding to erb-A polypeptides synthesized *in vitro*. Samples (2 μl) from the pA101(sense strand)-programmed reactions were used in ^{125}I -T₃ standard binding reactions²⁹ with increasing concentrations of unlabelled thyroid hormone or analogues to compete with labelled hormone. Specifically bound thyroid hormone is plotted against concentration of competitor compound. *c*, Competition of triiodothyronine isomers for ^{125}I -T₃ binding to erb-A polypeptides synthesized *in vitro*. Binding reactions were performed as above adding increasing concentrations of T₃ isomers. Thyroid hormone bound to erb-A is plotted on the ordinate. *d*, Competition of thyroid hormone analogues for ^{125}I -T₃ binding to 0.4 M KCl HeLa cell nuclear extracts. HeLa cell nuclei were extracted with a buffer containing 0.4 M KCl (ref. 29). The protein extract (25 μg) (determined by Bio-Rad protein assay) was mixed with 0.6 nM ^{125}I -T₃ in standard binding reactions with increasing concentrations of thyroid hormone and analogues^{29,30}.

Methods. Labelled ^{125}I -3,3', 5-triiodo-L-thyronine (NEN, 2,200 Ci mmol⁻¹, 0.3 nM final) was mixed with erb-A polypeptides synthesized in the *in vitro* translation mixture (described in Fig. 4) in T₃-binding buffer (0.25 M sucrose, 0.25 M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 2 mM EDTA, 5 mM dithiothreitol (DTT))²⁹ at 0 °C for 2 h in a final volume of 250 μl . Specific hormone binding was determined by adding a 1,000-fold excess of unlabelled hormone and assayed by counting radioactivity eluting in the excluded volume from a Sephadex G-25 fine (Pharmacia) 0.9 $\times$ 4.0 cm column²⁹.



stantiated by demonstrating its transcriptional regulation of T₃-responsive genes such as the growth hormone gene.

Analysis of the hGR and hER has revealed the proteins to be composed of a series of functional domains^{2-4,32-35}. These include a cysteine-rich region which contains structural similarity to a repeated cysteine-rich sequence found in *Xenopus* 5S gene transcription factor IIIA and other transcriptional regulatory proteins^{36,37} as well as carboxy-terminal sequences, which encode the steroid-binding domain^{3,4,38}. Extension of this analogy to the thyroid hormone receptor would predict its hormone binding region to be localized near the carboxy-terminal end of the molecule (Fig. 6). Putative DNA binding sequences would be found in the cysteine-rich region (Fig. 6) where DNA binding properties of the hGR and hER appear to be localized (refs 3, 38; S. Hollenberg and R.M.E., unpublished data).

Thyroid hormone receptor and oncogenesis

Expression of the *v-erb-A* product in avian erythroblasts is required for maintenance of the fully transformed phenotype⁴⁰⁻⁴³. Chickens infected with viruses lacking the *v-erb-A* gene display a less virulent disease, while *in vitro* these infected erythroblasts differentiate spontaneously and grow only with complex media supplements. Cells infected with *erb-A*⁺/*erb-B*⁺ virus, however, have an increased capacity for self-renewal and display a less differentiated phenotype. Structural alterations of the *v-erb-A* protein could give rise to a product exerting aberrant properties of growth control. For instance, changes at the carboxyl terminus might affect thyroid hormone binding activity, as has been shown for the β form of the human glucocorticoid receptor, resulting in a constitutively active molecule²⁻⁴, where changes abolish steroid binding activity. Insertional mutants in this domain also inactivate steroid binding properties³. However, deletion of the hormone binding region gives rise to a constitutively active receptor indicating that this domain plays a modulatory role in transcriptional activation (V. Giguere and R.M.E.,

unpublished data). These data lead us to predict that *v-erb-A* is unlikely to bind hormone and is rather a constitutively active form of the thyroid hormone receptor. The identification of *erb-A* as the thyroid hormone receptor provides the first direct evidence of a causative involvement of enhancers and their binding proteins in oncogenic transformation.

A superfamily of regulatory genes

Similarity of the steroid receptors with the *v-erb-A* oncogene product was sufficient to allow us to propose that both have evolved from a primordial receptor gene⁴. Two surprising results have emerged from the studies presented here. The first is that

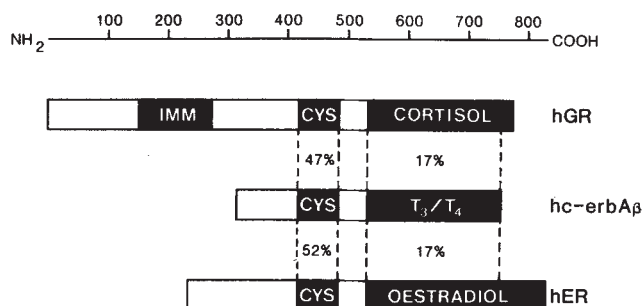


Fig. 6 Schematic comparison of the steroid and thyroid hormone receptors. Amino-acid sequences of the receptor molecules aligned in Fig. 2 are represented schematically. CYS, cysteine-rich region encoding the putative DNA binding domain found in the receptor proteins (Cys-rich region residues are: c-erb-A, 102-169; hGR, 421-486; hER, 185-250). Cortisol, oestradiol and T₃/T₄, hormone binding regions in the carboxyl termini; IMM, immunogenic region of the human glucocorticoid receptor. Numbers separating boxes, percentage amino acid identities between the receptor species in the intervals between the vertical broken lines; hGR, human glucocorticoid receptor; hER human oestrogen receptor; hc-erb-A β , human thyroid hormone receptor.

the occurrence of a family of *erb-A* proto-oncogenes implies the existence of one or more other molecules closely related to the thyroid hormone receptor. Physiological studies have not predicted the existence of a second class of thyroid hormone receptors and thus the characterization of this family may shed new light on mechanisms of developmental and homeostatic regulation. The second surprising observation from these results is the close kinship of the thyroid hormone receptors with the steroid hormone receptor family. This relationship indicates that these molecules may all be part of a superfamily of regulatory proteins that have arisen over evolutionary time to match the increasing developmental and physiological demands of more complex eukaryotes. Thus, the putative identification of the *erb-A* protein as the thyroid hormone receptor leads to new insight into these issues and should provide an important tool

for analysis of the molecular basis of transcriptional activation by regulatory proteins.

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LETTERS TO NATURE

Rotational period of comet Halley

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The rotational period of the nucleus of comet Halley is a basic datum which must be accurately known if the vast collection of observations of this comet recently obtained from spacecraft and ground-based observatories are to be correctly interpreted. Sekanina and Larson, who have studied the morphology of the comet's coma, find a period near 2.2 days, as do investigators associated with the Planet-A, Vega 1 and Vega 2 imaging experiments¹⁻⁴. Others, however, have reported significantly different values, or have been unable to find clear evidence of periodicity in their data⁵⁻⁷. We report here the results of ground-based photometry of comet Halley carried out during March and April 1986 which indicate that the rotational period of the comet is, in fact, near 7.4 days.

During an extended photometric study of comet Halley during the 1985-86 apparition, two lengthy observing runs were scheduled on the 0.61-m Lowell-Tololo telescope at Cerro

Tololo Inter-American Observatory (CTIO) in March and April of 1986. The March run (4-18 March) was timed to span the encounters of the Vega, Planet-A, and Giotto spacecraft with the comet, while the April run (29 March-19 April) covered the time of the comet's closest approach to Earth. Of the 37 nights on which we had use of the telescope during the March and April runs, 36 were, at least in part, photometric. Consequently, much high-quality photometric data were obtained. While our main aim was to measure production rates of various gases and dust and to determine their variation with heliocentric distance, it quickly became apparent that the comet was undergoing sizeable night-to-night changes in activity.

The observations were made using a conventional single-channel photometer equipped with pulse-counting electronics. Interference filters, mostly chosen from the standard IAU set⁸, were used to isolate emission bands of OH, NH, CN, C₃, CO⁺, and C₂ along with continuum regions at 3,650 and 4,845 Å. Observations were made with the comet's apparent photocentre centred in the entrance aperture of the photometer. Each observation typically consisted of two or three 10-s integrations on the comet minus a sky measurement made several degrees away. Extinction corrections were determined nightly and have been applied in the standard fashion. The observed intensities were then transformed to absolute fluxes by reference to nightly observations of several standard stars. Finally, the underlying

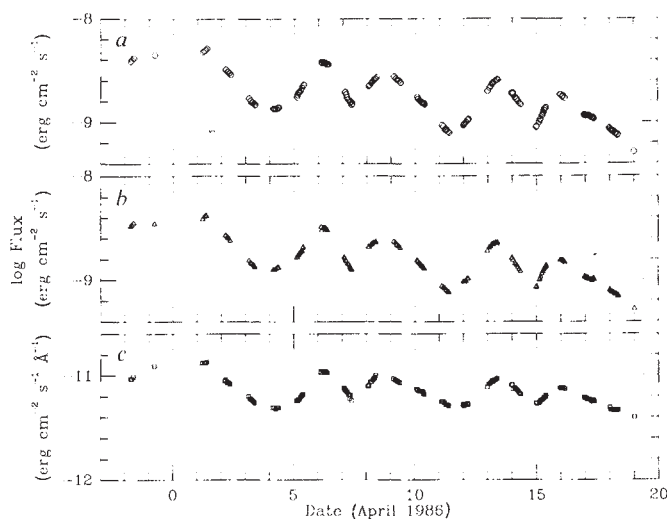


Fig. 1 Brightness variations in the inner coma of comet Halley seen during April 1986 in the light of emission bands of: *a*, C_2 (5,140 Å); *b*, CN (3,870 Å); *c*, in a continuum passband at 3,650 Å.

continuum within the emission-band filters, based on the continuum-band results, was subtracted.

Figure 1 shows the flux from comet Halley through selected passbands measured during the April run. These observations were made with a photometer entrance aperture of 39-arc s radius. Light curves of essentially identical shape were recorded through the other filters and with entrance apertures of different sizes. The observational uncertainty in each plotted point is comparable to or less than the size of the point. It is evident from a brief inspection of Fig. 1 that the brightness of the central region of the coma was varying by a factor of ~ 4 on a timescale of a few days.

For brevity, we will discuss only observations made in a single passband centred on an emission band of C_2 at 5,140 Å and with the same 39-arc s radius aperture. This aperture, which is the one we used most frequently, had a corresponding radius at the nucleus of the comet which ranged between 33×10^3 and 12×10^3 km during March and April 1986. To remove, at least

to first order, the effects of the variation in aperture size at the comet, we have converted the observed C_2 fluxes to column densities and then to production rates assuming a Haser model. While one might quibble with the applicability of the Haser model to comet Halley, the results presented here concerning the short-period variability of the comet in no way depend on the strict validity of this model.

The observed production rates of C_2 in comet Halley during March and April are plotted as a function of time in Fig. 2. Because the comet was observable for most of each night in April, the continuity of the data from that month is sufficiently good that there is no question about the timescale of the variation. Furthermore, it is evident from even a casual inspection of Fig. 2 that the photometric behaviour of the comet between 3.5 and 11 April was repeated almost exactly between 11 and 18.5 April, indicating that the light curve has two maxima per cycle with a period near 7.5 days. During the March run, the period was evidently similar, but the light curve had a more complex shape than in April. Contemporaneous International Ultraviolet Explorer (IUE) observations with the FES (fine error sensor) camera⁹ agree with the shape of the mean March light curve as we have drawn it through the observed points.

In addition to the periodic variation, a steady secular decrease in the production rate of C_2 can be seen in Fig. 2 throughout March and April. This secular change, which presumably resulted from the decreasing insolation as the comet moved away from the Sun, has been removed from the data before performing the period search described below.

A search for periodicity in the observations was performed by applying the method of phase dispersion minimization separately to the March and April data. This method¹⁰ is ideal for application to data such as those in Fig. 2 because it makes no assumptions about the shape of the light curve. In this method, the data are phased according to an assumed period and then divided into a number of phase bins, M , spanning the full phase interval. The variance within each bin, S_j^2 ($j=1, M$), is computed; and then the overall variance for all bins S^2 is calculated from

$$S^2 = \frac{\sum (n_j - 1) S_j^2}{\sum n_j - M}$$

where n_j is the number of data points in the j th bin. A statistic, θ , defined as the ratio of S^2 to the variance of the total data set, is computed. This process is repeated for a broad range of trial periods. If the assumed period is very different from the true period, the phased data will constitute a scatter diagram and $\theta = 1$. At a true period, however, θ will reach a local minimum.

In Fig. 3a, we have plotted θ as a function of period for the April data. In computing θ , the data were divided into five phase bins. Note that over the entire range of periods from 1 to 10 days, the deepest minimum is at ~ 7.4 days. There is no significant minimum near 2.2 days. Figure 3b, c shows a plot of θ against period at higher resolution for the March and April observations. For the April data, a sharp minimum is found at 7.37 ± 0.07 days. The quoted uncertainty is estimated. Because of less complete sampling of the light curve in March as compared with April, the minimum centred at 7.41 days in the θ versus period plot has a broad, flat bottom. The uncertainty in the period determination based on the March data alone is estimated to be ~ 0.1 day. Another minimum at 3.9 days was found in the analysis of the March observations. However, that period, which is almost exactly four times the sampling interval, can be shown to be an artefact of the period-finding method.

Composite light curves for March and April, constructed by phasing the data according to the 7.37-day period, are plotted in Fig. 4. We have adopted this single period for the two data sets because the two values determined above are the same to within their estimated errors and because we feel that the April run provided the more accurate value. The superposition of

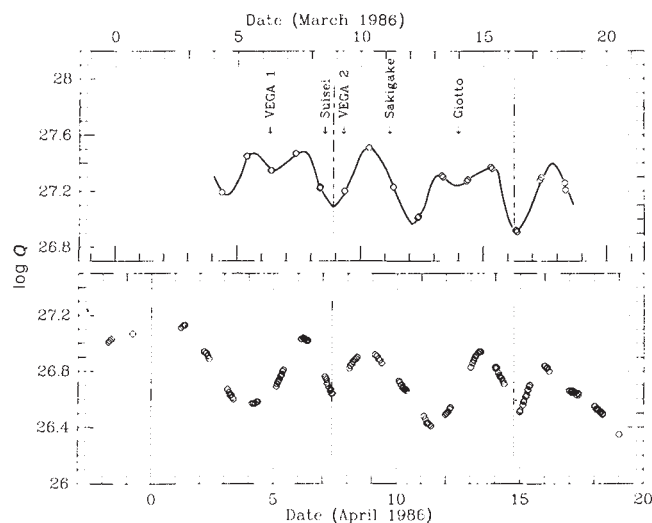


Fig. 2 Production rate of C_2 as a function of time in March and April 1986. Arrows mark the times of the various spacecraft encounters. Dashed lines identify corresponding minima in March-April.

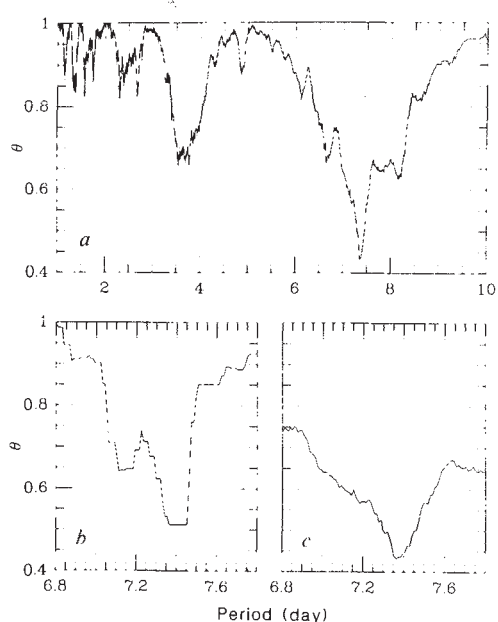


Fig. 3 *a*, The statistic θ plotted as a function of period for the data between 29 March and 19 April. At a true period, θ is expected to show a local minimum. By far the deepest minimum seen here corresponds to a period near 7.4 days. *b* And *c*, the same type of plot as described above shown at expanded time resolution for the 4–18 March and 29 March–19 April data, respectively.

successive cycles is surprisingly good. Certainly, one would not expect the light curve of an active comet to be as repeatable as that of an inert object like an asteroid; and, indeed, the form of comet Halley's light curve did evolve between the two observing runs. The abscissae in Fig. 2 have been located so that the March data are placed relative to the April data, as if shifted forward in time by four cycles of the 7.37-day period. Note that the minima marked by the dashed lines nearly coincide in phase. In fact, the primary change in the light curve between March and April is the absence in April of the first hump of the broad maximum seen in March. A change of this character in the shape of the light curve could easily occur if a localized region of activity on the comet's nucleus became dormant or, at least, substantially decreased its output during the last 2 weeks in March. The underlying periodicity of the light curve, on the other hand, would be unaffected.

We believe that the most natural explanation of the periodic variations that we have observed in the brightness of comet Halley's inner coma is an up-and-down fluctuation in the comet's overall level of activity as surface regions of greater or lesser volatility are brought into sunlight by the rotation of the nucleus. The observed period of the brightness variations is, therefore, identified with the synodic period of rotation of the cometary nucleus. If this interpretation is correct, the observed period would be expected to have decreased by ~ 0.1 day between March and April if the sense of rotation is the same as that of the orbital motion. Such a change is consistent with our observations. However, our period determinations are not sufficiently accurate to unequivocally confirm a change of this magnitude.

Comet Halley's light curve is seen to have basically two maxima and two minima per rotation. While not required, such a light curve is not unexpected given the shape of the nucleus as revealed by the Vega and Giotto imaging experiments^{11,12}. Comet P/Arend-Rigaux, whose nucleus also has been shown to be elongated, has a two-peaked light curve¹³.

Of the previous attempts to ascertain the rotational period of comet Halley, some, like the present study, were based on ground-based photometry, albeit with much less continuous observational coverage^{6,7}. The results have been inconclusive.

Whipple⁵ and Sekanina and Larson^{1,2} have taken a different approach based on the morphology of the coma as recorded primarily on ground-based photographs. Sekanina and Larson find a rotational period near 53 h. Investigators analysing observations from the Japanese ultraviolet imager experiment and the Vega 1 and 2 imaging experiments also have reported periods near that value^{3,4}. However, pending a fuller description of the spacecraft results, we are unconvinced that their period determination should be accepted as firm. The ultraviolet imager on the Planet-A Suisei spacecraft detected several brief Ly α flashes over a period of months. However, the total interval is extremely undersampled. Continuous coverage has been published for only one 2.4-day interval, during which several flashes were seen. If several flashes can occur per cycle and if the light curve has not been shown to repeat over even two successive cycles, then determination of a unique period would seem difficult. The Vega 1 and 2 investigators have measured the rotational period of comet Halley's nucleus by examination of images obtained during two 40-min observation periods separated by 3 days. While we in no way wish to denigrate the remarkable accomplishment that the Vega 1 and 2 images represent, they are of low resolution. It seems questionable that a truly unique period determination could be based on those images.

Given the strong periodicity at 7.4 days in the photometric data reported here and the absence in these observations of evidence for other periods, we believe that previous determinations of the rotational period of comet Halley should be re-examined. We can think of no source of the variations present in our data other than rotation of the nucleus. If this interpretation of the photometric data is correct, then we can predict the relative orientation of the nucleus at the times of the encounters of the comet with the Planet-A, Vega 1 and 2, and Giotto spacecraft. The times of these encounters are indicated by arrows in Fig. 2. We expect that at the Vega 1 and Giotto encounters the orientation of the nucleus with respect to the Sun was very similar. At the time of the Vega 2 encounter, however, roughly the opposite side of the nucleus was facing sunward. Suisei and Sakigake were not equipped to image the nucleus.

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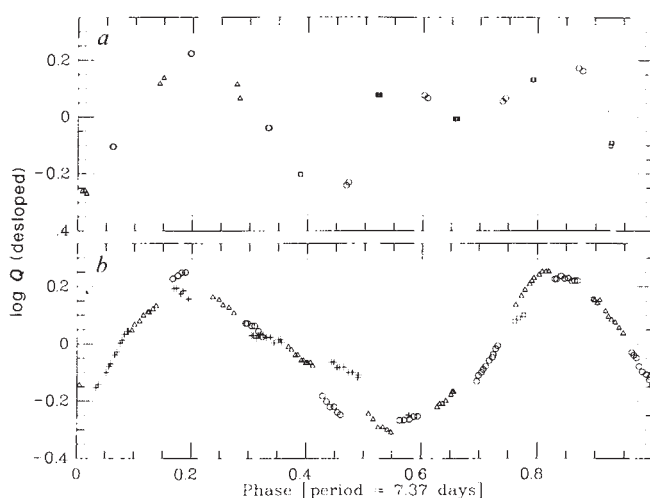


Fig. 4 Composite light curves of comet Halley constructed by phasing the data in Fig. 2 according to a 7.37-day period. *a*, 4–18 March; *b*, 29 March–19 April. The secular decrease in the mean brightness of the comet evident in Fig. 2 has been removed before plotting. Successive cycles of the variation are plotted with different symbols. The different cycles are delineated by dashed lines in Fig. 2 and are plotted here in order of increasing time as squares, circles, triangles, and pluses.

with the observations in April, and Tobias Kriedl provided software for the period search, as well as helpful discussion of the phase dispersion minimization method. This research was supported by NASA grant NGR-03-003-001.

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Cyanogen jets in comet Halley

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Current theory holds that jets, spirals and other detailed features in cometary comae are visible only in the continuum reflected by the dust particles. It has generally been thought that these features could not be seen in the emission bands of the typical neutral species, at least in part because the neutral parent molecules expand in directions perpendicular to any linear feature in a time short compared with that required to travel the length of the features. Consequently, the daughter species normally observable at optical and ultraviolet wavelengths are produced by dissociations that take place far from the point of origin of the gas. Here we present images of comet Halley taken in the light of CN emission, that show prominent jets extending to >60,000 km from the nucleus and lasting several weeks. We interpret these images as jets of submicrometre particles, perhaps the organic 'CHON' particles detected from spacecraft, from which the CN is directly produced.

During comet Halley's post-perihelion phase, we carried out observations with the 24-inch planetary patrol telescope at Perth

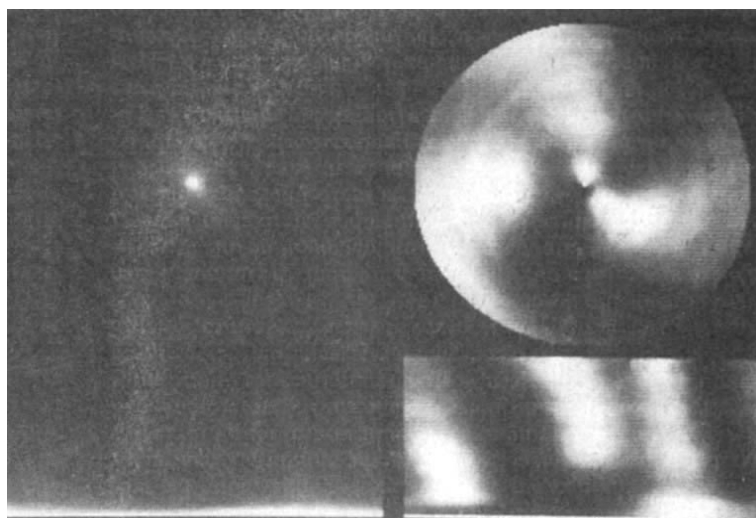
Observatory, using a CCD (charge-coupled device) camera system which is one of two developed jointly with S. M. Larson (the system will be described elsewhere). The present observations were made in April, when the comet was near opposition, with an effective focal ratio of 5.4 and a scale of 1.8 arc s per pixel on the CCD chip. The filter set is a duplicate of the IAU standard filters distributed to the photometry and polarimetry net of the International Halley Watch and the photometric calibration was done with exactly the same techniques as used by that net.

The comet was imaged several times each night in various emission-band and continuum filters. Jets were visible in real time on the television monitor in the raw images taken with the CN filter. These features are not visible in nearly simultaneous images taken in the reflected continuum, nor are they coincident with the sharper, shorter features seen by shift-difference processing in nearly simultaneous broad-band images processed by S. M. Larson (personal communication). The jets seen in CN light appear to be of much higher contrast, broader, longer and in totally different orientations.

Figure 1 shows the processing that has been applied to all subsequent images. At the upper left is a direct image taken on 23 April, corrected by bias subtraction and flat field and smoothed with a three-point triangle (Hanning function). In all images presented here, north is at the bottom and east is at the left. This image, only a portion of the total frame from the CCD, is 5 arc min = 10^5 km square. The existence of three jets is clear: they extend roughly south, east and north-west but curve substantially into spirals. Images in the continuum have not yet been subtracted from our images in CN but photometric calibration indicates that the continuum will be negligible except in the few pixels nearest the nucleus. For processing, the image was extracted to an (r, θ) plot as shown at the lower left, in which radius is the ordinate and azimuth is the abscissa. To enhance the jets each horizontal line of the (r, θ) representation was renormalized to the maximum and minimum values along that line. The result is shown at the lower right, in which the shape of the jets is much more clearly seen than in the unnormalized representation. This plot was then converted back to the conventional image format at the upper right. This procedure was followed for all of the images shown in Fig. 2. All images were also scaled to the same physical size in kilometres at the comet.

It is clear that the jets have a spiral shape due to the rotation of the nucleus; despite the first impression, however, the view is not along the polar axis. Figure 2 shows a series of images from 16-23 April. During this period, the northern rotation pole (that is, the angular momentum vector) as defined at the 1910

Fig. 1 Image processing steps applied to all images. The upper left panel is the cometary image extracted from the original CCD image, no. 81 in Table 1. Bias subtraction and flat-field corrections have been applied. The lower left panel is a radius (r) against polar angle (θ) display of the original image. The radial distance increases upward and the position angle on the sky is the abscissa varying from west through north. The lower right panel is a contrast enhancement of the lower left panel. The enhancement is such that each line, a circle in the original image, has been scaled between the minimum and maximum of the display. This effectively removes all radial variation. The upper right panel is the enhanced (r, θ) plot converted back to an (x, y) coordinate system.



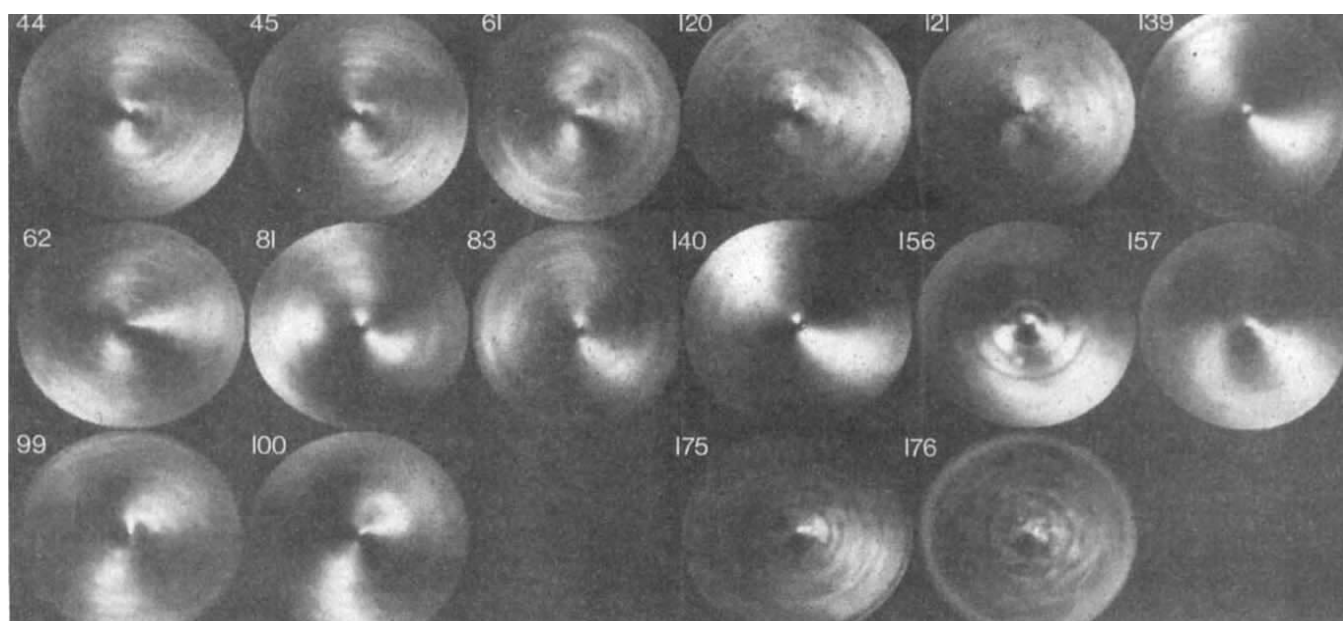


Fig. 2 The radially enhanced images for eight days at two frames per day. The details of the exposures are given in Table 1. Time increases with image number. Each image has undergone the enhancement process described in the text and Fig. 1.

apparition¹ was pointing nearly southward as seen on the sky and changed from nearly in the plane of the sky at the beginning of the period to pointing towards Earth, $\sim 20^\circ$ out of the plane of the sky. Preliminary indications from the present apparition are that the current rotation pole may differ by $\sim 20^\circ$ from that determined in 1910^{2,3}. The position angle of the Sun varied across the entire south-west quadrant, from 199° on 16 April to 272° on 29 April.

Identification of jets persisting from one day to the next is dependent on the assumed period of rotation. Examination of the series of images, many of which are shown in Fig. 2, shows that there are at least three and, if the rotation period is 2.17 days¹, probably five jets involved. It is possible that other rotation periods may be consistent with the pattern, perhaps requiring fewer jets. A more detailed analysis will follow.

The ratio of the ejection velocity of the material in the jets to the rotational velocity of the nucleus can be estimated from the slope of the jets in the (r, θ) plots. With the assumptions that the spiral patterns are due to projected helices and that the rotational period is 2.17 days¹, the ejection velocity is $\sim \frac{2}{3}$ km s⁻¹, comparable to that inferred from spacecraft^{4,5}. This scales inversely as the rotational period, but is only an order of magnitude estimate. Again, detailed modelling will follow.

The fact that the jets are so pronounced in the light of CN has significant implications for the formation of CN radicals. Figure 3 shows brightness profiles as a function of azimuth for several different radii in the image shown in Fig. 1: these represent horizontal cuts through the lower right panel and they show a range of 720° in azimuth (two full rotations) to exhibit more clearly features that would be at the edge of a plot showing only one rotation. The full width at half maximum (FWHM) of the CN distribution is $\sim 45^\circ$ at a radius of 30,000 km, which corresponds to a linear FWHM of 24,000 km. The emission from the jet must be due to fluorescence rather than, for example, prompt emission (emission immediately following dissociation into the excited $B^2\Sigma$ state) because a typical CN radical will fluoresce many times before leaving the jet, whereas it can produce only one photon by prompt emission. The half width at half maximum of the parent, then, must be $\leq 3,000$ km, much too narrow for the parent to be a neutral gaseous species released from the nucleus. If we take the FWHM of the parent species to be 3,000 km at a radial distance of 30,000 km and take $\frac{2}{3}$ km s⁻¹ as the ejection velocity, the maximum transverse velocity corre-

sponds to a temperature near 5 K. In the absence of a confining mechanism for the jets, the inescapable conclusion is that the jets are composed of dust grains and that the CN is either dissociated directly from molecules on the surfaces of the grains or dissociated from parent molecules that have a very short ($\tau < 1,000$ s) lifetime before photodissociation and that vaporize from the grains. Although this result was quite surprising to us, Bobrovnikov^{6,7} reported such jets in comets Halley and Westphal, and Wurm and Mammano⁸ discussed the rapid formation of CN in expanding haloes.

Why do we not see these dust grains in the images taken in the continuum? As pointed out by Sekanina³, the grains are probably no larger than $0.1 \mu\text{m}$, so they would not be seen in the reflected continuum, which is dominated by much larger grains. In fact, the spacecraft that passed through the coma of Halley detected many more very small particles than had been expected^{4,5,9,10,11}. It seems likely that the CN is being dissociated directly from the grains because estimates of the dissociation lifetimes of a variety of plausible parents for CN are all $> 10^4$ s (ref. 12). It is tempting to suggest that the CN comes from the organic 'CHON' particles found by the spacecraft^{13,14}.

Table 1 Exposure data for selected CN imagery of comet Halley

Image no.	Date	Exposure		<i>R</i> (AU)	Δ (AU)
		Start (UT)	Duration (s)		
44	86.4.16	17:29:44	600	1.42	0.46
45		18:32:38	600		
61		14:05:46	600		
62	86.4.17	16:01:04	900	1.43	0.48
81		12:39:05	900		
83		17:16:39	900		
99	86/4/24	13:23:51	900	1.54	0.63
100		14:53:00	1,200		
120	86/4/26	11:36:32	1,200	1.57	0.68
121		15:08:45	1,200		
139		15:25:37	1,200		
140	86/4/27	17:46:04	1,200	1.58	0.71
156		11:04:54	1,200		
157		13:45:19	1,200		
175	06/4/29	11:52:06	1,200	1.61	0.76
176		14:25:59	600		

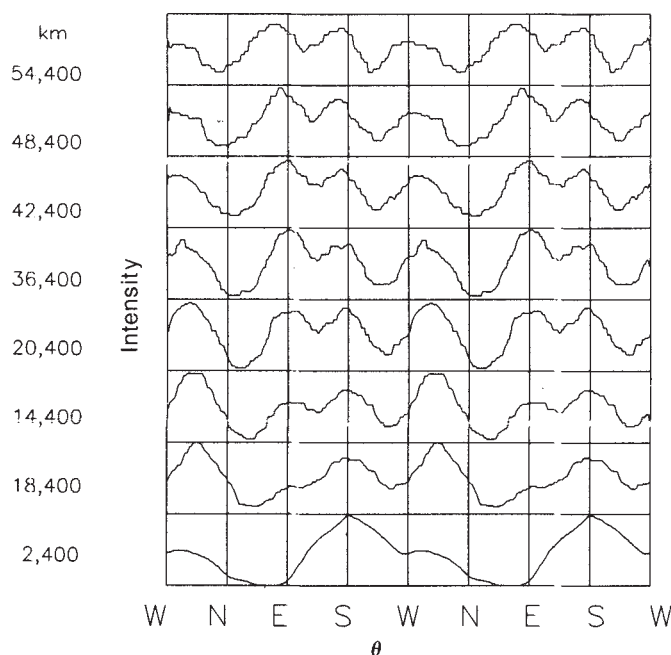


Fig. 3 Plot of normalized intensity against azimuth for eight discrete radii projected on the sky, labelled by the value of projected radius. These plots are horizontal cuts through the lower right panel of Fig. 1, image no. 81 in Table 1.

A very crude estimate of the fraction of the CN in the comet that is produced in this way can be obtained by examination of the unnormalized version of Fig. 3. At radii of 10,000–20,000 km, the fraction of the column density of CN that is in the jets is ~10–15%. Because the high ejection velocity of CN on photodissociation causes the CN in the jets to diffuse rapidly into the background, it is not unreasonable to assume that this represents the total fraction of all CN that comes from the grains.

It is not yet clear whether the CN jets discussed here and attributed to dissociation from grains are related to the fluctuations in CN detected from the spacecraft. Levasseur-Regourd *et al.*¹⁵ reported that an excess of CN (relative to a vectorial model) was detected near the nucleus by the Optical Probe Experiment on Giotto; they attributed this excess to prompt emission. In addition to the excess emission that they reported near the nucleus, examination of their Fig. 2 shows fine structure in the CN flux, particularly an enhancement near $\log r = 3.6$, which is clearly above the noise and might be due to passage of Giotto through jets such as those reported here. Similarly, Moreels *et al.*¹⁶ have reported that the three-channel spectrometer on Vega 2 also detected an excess of CN near the nucleus. The spacecraft data clearly merit a more detailed examination in the context of additional ground-based data. Our own images from the time near the spacecraft encounters do not show any prominent jets in CN, but those images have not yet been examined in sufficient detail to rule out the presence of such jets. It does seem at least plausible that the excess CN seen by the spacecraft experiments is provided by dissociation from the organic grains, as we have suggested for the CN in the jets reported here. If our hypothesis is correct, similar structures should be visible in images taken in the light of other radicals composed of 'CHON', provided that they have sufficient contrast with the background. Preliminary studies of images in the light of C_2 indicate that the jets are present but weaker, because of the strong contribution by the continuum which does not exhibit jets.

M.F.A'H. and S.H. thank the staff of the Perth Observatory and the University of Western Australia for hospitality and assistance during the course of these observations, particularly

Arie Verveer for his technical assistance, and the government of Western Australia for their support through these institutions. We thank Zdenek Sekanina and Nalin Samarasingha for several helpful discussions regarding the geometry of the jets and Z.S. for pointing out that the dust grains must be very small; Steve Larson for comparing these CN jets with the ones used in modelling the nuclear rotation, and for designing and building the CCD system; many individuals for comments regarding the relationship to the organic grains; Jürgen Rahe for pointing out the earlier work on CN structures; and Barbara Boothman for programming the algorithms for the (r, θ) extraction and providing general assistance with the computing. The CCD system was constructed with financial support from the International Halley Watch office at the Jet Propulsion Laboratory. The observing programme was supported by NASA grant NSG 7322 and NSF grant INT 85-14707 to the University of Maryland. *Note added in proof:* The existence of submicrometre 'CHON' particles in comets has recently been discussed by Greenberg¹⁷ as a consequence of accretion of interstellar grains.

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Nitric acid cloud formation in the cold Antarctic stratosphere: a major cause for the springtime 'ozone hole'

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Large depletions in stratospheric ozone were first reported by Farman *et al.*¹ at Halley Bay (76° S), and confirmed by satellite observations². Chubachi³ gives a detailed account of ozone decreases and temperatures in the lower stratosphere during the spring of 1982 at 69° S. There is now evidence² for annual declines in total ozone by ~6 and 3% in regions of total ozone minima and maxima, respectively, from September to mid-October since the late 1970s. We propose here a chemical mechanism for the formation of the ozone hole. It involves removal of gaseous odd nitrogen by ion- and/or aerosol-catalysed conversion of N_2O_5 and $ClONO_2$ to HNO_3 vapour, followed by heteromolecular HNO_3 - H_2O condensation, leading to HNO_3 - H_2O aerosols. At an altitude of 17 km, these processes start at temperatures below 205 ± 5 K, well above the condensation temperature of pure water vapour. We propose that the absence of gaseous odd nitrogen and catalytic methane oxidation reactions driven by sunlight in early spring lead to large OH concentrations which rapidly convert HCl to ClOX. Catalytic reactions of ClOX and BrOX cause drastic ozone destructions and can account for the springtime 'ozone hole' first observed by Farman *et al.*¹. By our model the depletion would be

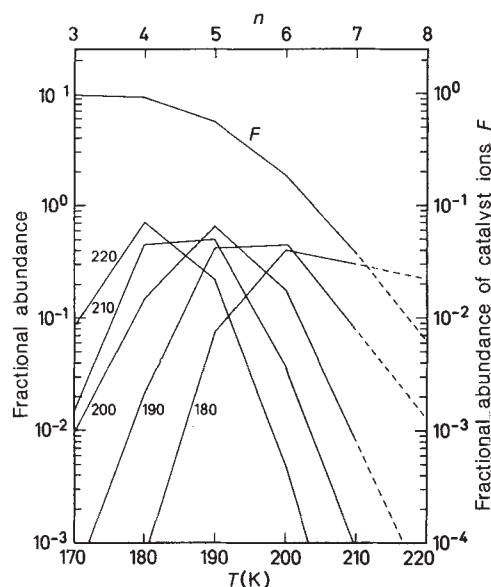


Fig. 1 Size distributions of $\text{H}^+(\text{H}_2\text{O})_n$ cluster ions (upper abscissa and left ordinate) for an altitude of 17 km, an H_2O -vapour volume mixing ratio of 3×10^{-6} and temperatures of 180, 190, 200, 210 and 220 K (thermodynamical data of Lau *et al.*¹⁷). Also shown is the fraction F of $\text{H}^+(\text{H}_2\text{O})_n$ ions (lower abscissa and right ordinate) with $n \geq 6$ that may serve as catalysts.

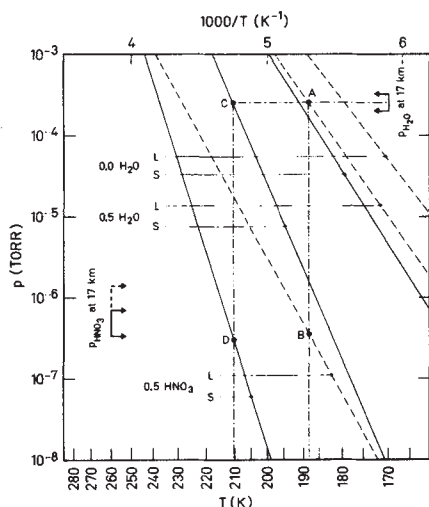


Fig. 2 Equilibrium saturation vapour pressures of H_2O and HNO_3 over solid (solid curves) and liquid (broken curves) H_2O - HNO_3 mixtures with HNO_3 mass fractions of 0.5 and 0.0 (pure H_2O). Also shown are partial pressures for H_2O and HNO_3 as observed in the background atmosphere around 17 km altitude²⁹. Condensation temperatures for liquid mixture (points A and B) and solid (points C and D) mixtures are about 188 K and 211 K respectively. For liquid and solid H_2O ($f_2 = 0.0$) condensation temperatures are about 179 K and 191 K respectively.

mainly due to emissions of industrial organic chlorine compounds. Arctic regions may also become affected. The depletion lasts while HNO_3 , but not HCl , is incorporated in the particles in the temperature range 205 ± 5 K to 192 K.

Farman *et al.*¹ connected the ozone decrease with the extremely cold temperatures and stable dynamics of the lower stratosphere and also proposed that the depletion was due to the increasing transfer of industrial chlorine compounds to the stratosphere. This idea was later developed by McElroy *et al.*⁴ and Solomon *et al.*⁵. McElroy *et al.*⁴ consider reactions between ClO and BrO . Their scheme requires the absence of NO and NO_2 from the stratosphere, brought about by reactions of N_2O_5 and ClONO_2 on the surfaces of the stratospheric cloud particles

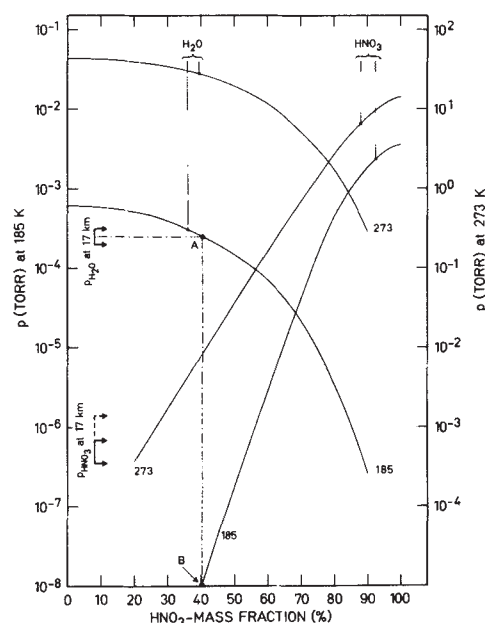
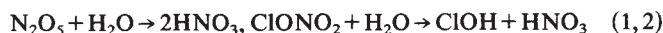
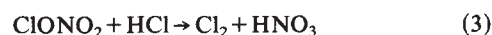


Fig. 3 Equilibrium saturation vapour pressures of H_2O and HNO_3 over liquid H_2O - HNO_3 mixtures against the HNO_3 -mass fraction f_2 for temperatures of 273 K and 185 K. Also shown are partial pressures of H_2O and HNO_3 as observed in the background atmosphere around 17 km altitude. Both sets of curves show the lowering of equilibrium vapour pressures of H_2O and HNO_3 over those of the one-component systems. At 185 K and $f_2 = 40\%$ large supersaturation of gaseous HNO_3 occurs and co-condensation of HNO_3 and H_2O takes place.

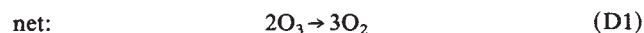
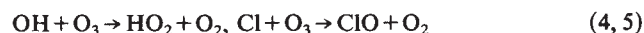
observed^{6,7} in polar regions:



and furthermore the conversion of most HBr to BrO_x and most HCl to ClO_x . For the latter assumption, however, they do not present a mechanism. Solomon *et al.*⁵ assume that efficient reactions of ClONO_2 and HCl occur in polar clouds:

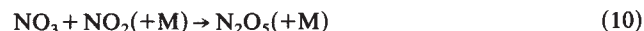
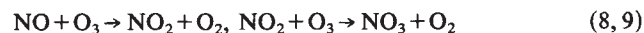


leading to the catalytic reaction chain



The efficiency of the heterogeneous reactions (1)–(3) for stratospheric conditions has not been measured, so the above studies are speculative.

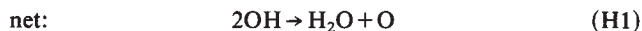
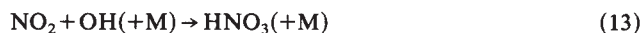
To explain the observations we present a chemical mechanism, involving the following steps: (i) conversion of NO_x to N_2O_5 during polar night:



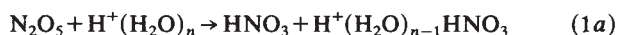
(ii) formation of HNO_3 by ion- and /or aerosol-catalysed conversion of N_2O_5 and ClONO_2 at temperatures ≤ 215 –210 K; (iii) heterogeneous, heteromolecular condensation of HNO_3 vapour with H_2O giving rise to stratospheric cloud particles and depletion of gaseous HNO_3 at temperatures $\leq 205 \pm 5$ K; (iv) efficient hydroxyl radical formation by cosmic ray ionization and ozone photolysis, amplified by photochemical methane oxidation reactions after sunlight returns to the polar regions; (v) conversion of HCl to ClO_x , and HBr to BrO_x , by reaction with OH ; (vi) ozone destruction through ClO_x and BrO_x catalysis.

Conversion of N_2O_5 and ClONO_2 to HNO_3 and the depletion of gaseous NO_x and HNO_3 , which are possible only at low temperatures, are key elements in our mechanism as they prevent

OH removal by the otherwise most important catalytic reaction cycle:



Conversion of N_2O_5 and ClONO_2 to HNO_3 via (1) and (2) may be catalysed by ions and/or aerosols. The ion-catalysed mechanism involves an H_2O molecule as a reaction partner, contained in a cluster ion⁸⁻¹⁰. Atmospheric ambient ions present at ~ 17 km, the centre of the height region of interest, are $\text{H}^+(\text{H}_2\text{O})_n$, $\text{H}^+(\text{CH}_3\text{CN})_m(\text{H}_2\text{O})_n$, and $\text{NO}_3^-(\text{HNO}_3)_m(\text{H}_2\text{O})_n$ as was found from recent balloon-borne mass-spectrometer measurements¹¹⁻¹³. For $\text{H}^+(\text{H}_2\text{O})_n$ ions, for example, N_2O_5 conversion may proceed via



becoming exothermic only for $n \geq 6$. Ions of the type $\text{NO}_3^-(\text{HNO}_3)_m(\text{H}_2\text{O})_n$ may be even more efficient catalysts as extra energy is gained due to stronger clustering of HNO_3 formed by the N_2O_5 reaction¹⁰. However, to serve as catalysts both positive and negative ions must be hydrated enough. This is possible only at low atmospheric temperatures. Fig. 1 shows calculated relative abundances of the members of the $\text{H}^+(\text{H}_2\text{O})_n$ family and also the fraction F of $\text{H}^+(\text{H}_2\text{O})_n$ ions with $n \geq 6$. Taking the calculated F values, lower limits to the N_2O_5 lifetime, t_i , against ion-catalysed conversion can be estimated. For a total ion concentration of $\sim 10^4 \text{ cm}^{-3}$ and a typical rate coefficient for an ion-molecule reaction of $2 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$ one obtains $t_i \geq 5 \times 10^4 / F$ s. Hence, at temperatures below about 215 K, t_i

may become shorter than one month. It should be mentioned, however, that exothermicity does not necessarily guarantee a large reaction coefficient and t_i may therefore be larger than this. For a more detailed discussion of ion processes see ref. 10.

Conversion of N_2O_5 and ClONO_2 on aerosols is rather uncertain as the reaction probability per collision, γ , is not well known. The lifetime t_{AC} of a gaseous molecule against collision with aerosols in the background atmosphere at ~ 17 km is $\sim 10^4$ s and thus the reaction time t_A becomes t_{AC}/γ . To make t_A one month, $\gamma = 2 \times 10^{-3}$ is required, which is much larger than γ measured¹⁴ for concentrated sulphuric acid at 300 K.

In the cold Antarctic stratosphere, however, the aerosol content may become much larger than in the background stratosphere as found from solar extinction measurements^{6,7}. This excess aerosol, termed polar stratospheric clouds (PSCs) has previously⁷ been explained in terms of H_2O condensation alone. Here we shall argue that heteromolecular condensation of HNO_3 and H_2O occurs and leads to a great increase of the aerosol mass and surface area, which, in turn, may accelerate aerosol-catalysed N_2O_5 and ClONO_2 conversion, and more importantly leads to a depletion of HNO_3 vapour. Figure 2 shows extrapolations of measured¹⁵ equilibrium saturation vapour pressures e_1 (H_2O) and e_2 (HNO_3) over an HNO_3 - H_2O mixture with an HNO_3 -mass fraction $f_2 = 0.5$. The extrapolations contain considerable uncertainties, especially for the solid state¹⁰. The case $f_2 = 0.5$ was chosen as this mixture gives the highest condensation temperature. Also shown in Fig. 2 are partial pressures for a_1 (H_2O) and a_2 (HNO_3) as found in the background atmosphere around 17 km altitude²⁹. The procedure to derive such conditions is illustrated in Fig. 3 for liquid mixtures and a temperature of 195 K. The condition for heteromolecular condensation ($e_1 \leq a_1$ and $e_2 \leq a_2$) for $T = 185$ K is met at f_2 near 40%. For this mixture $e_1 = a_1$, but $a_2 \gg e_2$,

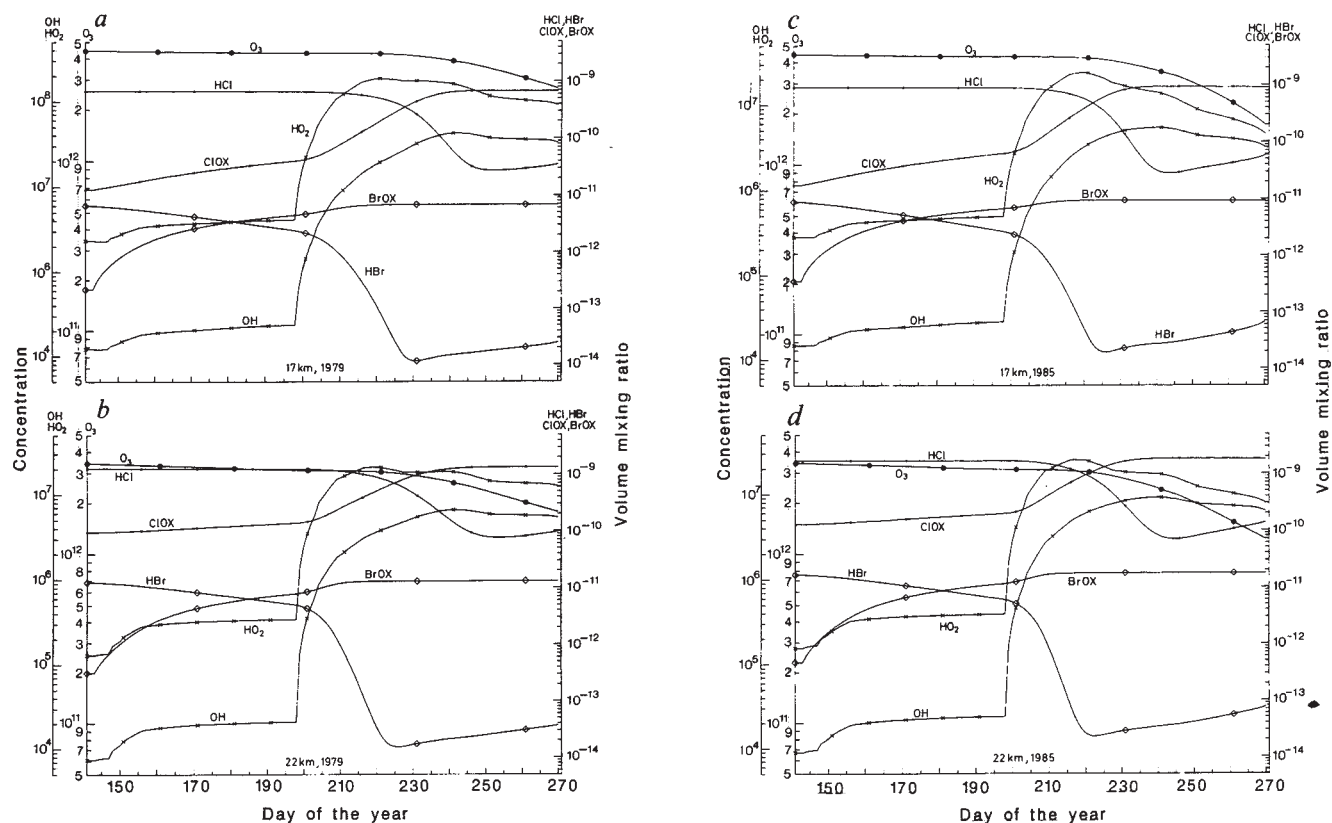


Fig. 4a, b, calculated concentrations (molecules cm^{-3}) at noon, of OH and HO_2 (first scale on the left) and of O_3 (second scale on the left), and volume mixing ratios of HCl, ClOX, HBr and BrOX (right scale). Polar night conditions at 70°S , here defined as Sun below horizon for 24 h period, lasted from 22 May (day 142) to 18 July (day 198). It was assumed that from day 147 nitric acid became incorporated in the polar stratospheric clouds. Calculations were performed for two altitudes, 17 km (a) and 22 km (b), and estimated conditions for the year 1979. c, d, Similar calculations as a, b, for 1985.

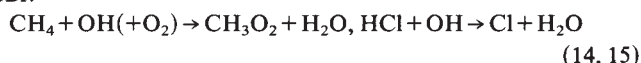
so that gaseous HNO_3 condenses out. This lowers both e_1 and the atmospheric partial pressure of HNO_3 and increases e_2 , so that about 1.5 times more H_2O than HNO_3 molecules condense out. Because $a_1 \gg a_2$, the resulting atmospheric pressures of HNO_3 will be much reduced, while those of H_2O are hardly affected.

An inspection of Fig. 2 reveals that the condensation temperature T_c for a liquid mixture with $f_2 = 0.5$ (points A and B) is about 188 K. For solid mixture one obtains $T_c = 211$ K (points C and D). A further cooling below T_c by only 3–4 K would be sufficient to deplete 50% of the HNO_3 vapour, giving rise to an increase of the aerosol mass and surface area by factors of about 100 and 20 respectively. Condensation of pure H_2O vapour, which becomes possible only at temperatures below about 191 K (solid) to 179 K (liquid), gives rise to an additional strong increase of the aerosol mass. The heteromolecular HNO_3 – H_2O condensation mechanism proposed here offers an explanation for recent extinction measurements^{6,7} showing a strong increase of the stratospheric total extinction at 1 μm wavelength over Antarctica as temperatures fall below about 205 K, which is close enough to 211 K considering the uncertainty of vapour pressure extrapolations and height distribution of the aerosol. Below about 191 K also other trace gases, particularly HCl, may become incorporated in aerosols¹⁰. With the increased aerosol surface, aerosol-catalysed N_2O_5 and ClONO_2 conversion may become important, depending on γ . However, ion-catalysed processes may be more efficient. It is possible that they are the missing source of HNO_3 in the polar winter stratosphere required in recent studies^{10,18}.

Another ion process which has a role in polar-night stratospheric chemistry is hydroxyl radical formation by ion chemistry driven by galactic cosmic rays. The rate of this ion-assisted OH formation is one to two OH molecules per ionization event or ~ 40 –80 OH molecules $\text{cm}^{-3} \text{s}^{-1}$ at high geomagnetic latitudes. Because the galactic cosmic ray ionization rate varies with solar activity, an 11-yr modulation will be induced. A more detailed discussion of ion and aerosol processes will be given in ref. 10.

Modelling of stratospheric photochemical processes is complex and all details cannot be discussed here. Our model uses a standard set of chemical reactions^{20,21}. To emphasize the effects of chemistry alone, we adopted a very small eddy diffusion coefficient, because the south polar vortex seems to be such a stable dynamic system²².

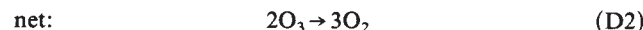
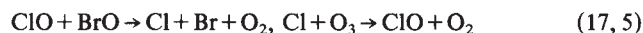
When gas-phase NO_x and HNO_3 are removed from the lower stratosphere, the OH radical reacts mainly with CH_4 , HCl and HBr.



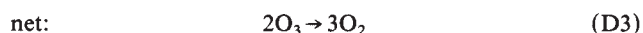
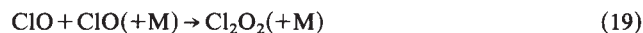
The results of the model calculations shown in Fig. 4, for altitudes of 17 and 22 km for 1979 and 1985, at 70° S, are mainly intended to demonstrate the sensitivity of ozone to odd-nitrogen removal during springtime. They are not close simulations of the exact conditions in the Antarctic. The calculations were started with concentrations obtained with our two-dimensional model. Ozone and temperature data were taken from the measurements³ made at Syowa (60° S) in 1983. The small jumps in the curves occurring around day 147 indicate the date at which we have assumed that HNO_3 becomes incorporated in the cloud particles. The large changes in concentrations which occur near day 200, about 20 July, mark the return of daytime conditions (Sun above the horizon) for some period during the day. Results are shown for noon. In these calculations CIOX denotes the sum of Cl, ClO, ClOH, Cl_2O_2 and OCIO. The last three compounds all dissociate rapidly in daylight, producing ClO_x (= Cl + ClO). BrOX is defined similarly. The stratospheric concentrations of total inorganic bromine were assumed to be 1% of those of inorganic chlorine, which seems to be roughly in agreement with the observations, although the uncertainty is

large^{23,24}.

Besides reaction cycle D1, there are two other catalytic reaction chains that are important in the breakdown of stratospheric ozone during early spring when sunlight has returned to the Antarctic:

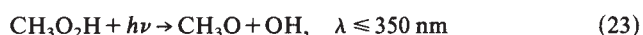


as considered by McElroy *et al.*⁴, and

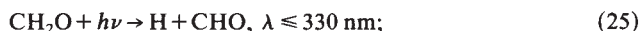


as recently introduced by Molina and Molina²⁵ and R. A. Cox (personal communication). According to Molina and Molina, the photolysis rate of Cl_2O_2 is roughly that of ClOH. The product yield of (20) is still only based on indirect evidence²⁵. For (19) we chose the rate expression $k = 6 \times 10^{-33} (300/T)^{2.1} \text{ cm}^6 \text{ molecule}^{-2} \text{ s}^{-1}$ by R. A. Cox (personal communication).

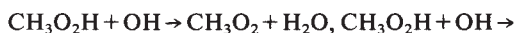
We note from the results in Fig. 4 that during polar night much HBr can be converted to BrOX by (16). The conversion would be much enhanced if HBr reacted rapidly with NO_3 , which seems quite likely. The conversion of HCl to ClOX is less efficient, because (15) is $\sim 1/25$ as fast as (16). During polar night, a large fraction of the OH radicals produced by the galactic cosmic rays is lost by reaction with CH_4 . The situation changes drastically after sunlight returns, when, starting from low odd hydrogen concentrations, reactions with CH_4 lead to rapid net production of odd hydrogen. The important reactions are:



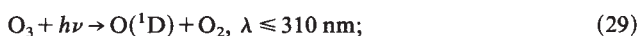
followed with about 30% probability by



The net result of the oxidation of one methane molecule by OH is therefore an average net gain of 0.6 odd hydrogen radicals, providing for a rapid buildup of OH radicals until these are also significantly lost by



For the first weeks, the main primary source of OH is production by galactic cosmic rays. Later, the reactions



become more important, further enhancing the OH concentrations. CIOX is efficiently produced from HCl after the polar night, leading to efficient destruction of ozone mainly through reaction cycle D1 and D3, with some contributions also from the $\text{BrO}_x/\text{ClO}_x$ cycle D2. The calculations for two altitudes 17 and 22 km, shown in Fig. 4, demonstrate the sensitivity of stratospheric ozone to the perturbations that characterize the

springtime Antarctic lower stratosphere, so that the observed trends in ozone removal can be explained by the chemical mechanisms presented above. The ozone decay continues as long as most HNO_3 is bound in the stratospheric polar clouds, that is, until the Antarctic stratospheric warming occurs in late September or early October^{3,22}. Then the evaporation of the polar clouds will reintroduce the various NOX gases into the lower stratosphere through known photochemical processes and most ClOX will be converted back to HCl. The decreasing ozone concentrations will delay the springtime solar warming of the lower stratosphere and this could gradually increase the period of unstable photochemistry. The ozone depletion will last as long as HNO_3 , but not HCl is incorporated in PSCs. At an altitude of 17 km this occurs between 205 ± 5 K and 192 K. Although less frequent than in the Antarctic, such conditions occur also in the Arctic stratosphere.

In this paper we could not present a detailed analysis of all features of the Antarctic ozone hole, because this requires consideration of and access to extensive meteorological, chemical and optical data sets²⁶. We have, however, clearly shown how in the Antarctic lower stratosphere during winter and springtime unstable photochemical conditions can develop that lead to drastic destruction of ozone. Several other complications that are likewise not treated in this paper must be considered in future studies, such as the effects of the major eruptions of volcano El Chichon in 1982, the possibility of gravitational settling of the particles²⁶, and the reactions of odd hydrogen and other chemical compounds on the particles.

To test our chemical mechanism, measurements of odd nitrogen and chlorine gases as well as ambient ions would be very informative. No measurements of HNO_3 and HCl have so far been reported in the Antarctic for September–October. Available total column NO_2 observations^{27,28} agree with the mechanism proposed, but do not provide proof. Also laboratory studies of the binary H_2O – HNO_3 system at low temperatures and ion-catalysed reactions are needed.

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Are domains in liquid crystalline polymers arrays of disclinations?

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Interaction of mobile disclinations in the liquid crystalline state results in various disclination arrays. The mesophase state of liquid crystalline polymer (LCPs) samples has been assumed to consist of a polydomain texture, each domain having an internal structure of uniform orientation bounded by regions where the molecular director field undergoes reorientation from one domain to its neighbour. However, the detailed nature of the boundary region (in particular whether the molecular director reorients in a continuous or discontinuous fashion) is largely unknown. We have observed groupings of disclinations of alternating sign which resemble Bouligand's serrated wall structure¹ and 'foursomes' of two positive and two negative disclinations which bear a likeness to the 'effective domain' structure recently hypothesized by Marrucci² to account for certain rheological aspects of LCPs. The arrangements of disclinations in our thin-film samples give rise to an apparent polydomain texture consisting of regions of uniform molecular orientation bounded by disclinations where the molecular director undergoes continuous rotation about an axis parallel to the disclination lines (Neel walls). The specific disclination arrays observed will be influenced by the molecular weight and stiffness constants of the particular LCP and by the flow history, thickness and boundary conditions of the film³; nevertheless, except in the vicinity of the disclination cores, the field of molecular director trajectories throughout the polydomain sample texture is both continuous and translationally parallel.

Due to industrial and academic interest in stiff chain and semiflexible polymer molecules, there have been an increasing number of structural and rheological studies of liquid crystalline polymers in recent years. A banded texture is often noted in flow-oriented LCP specimens. Donald *et al.*⁴ have examined the banded structures in detail and have recently shown that annealing transforms the banded morphology into a 'vein' structure which they relate to orientation domains. Initial explanations for phenomena such as the yield stress and the three-region viscosity-shear rate curve (two shear-thinning regions separated by a newtonian plateau) have invoked the polydomain concept where, during flow, the domains slide by one another easily at low shear rates. At moderate shear rates, the domains are supposed to break up, leaving dispersed domains in matrix of uniform molecular orientation, and, at high shear rates, a continuous monodomain structure is realized⁵. A recent theory of Marrucci², however, emphasizes the role of disclinations in the rheology of liquid crystalline polymers. The fluid is imagined

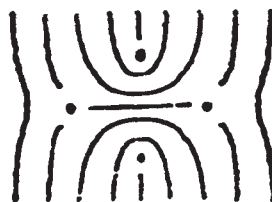


Fig. 1 Schematic of an effective domain in a liquid crystalline polymer consisting of an array of four half-integer disclinations. Lines indicate the molecular trajectory about the defect lines (●) (from ref. 2).

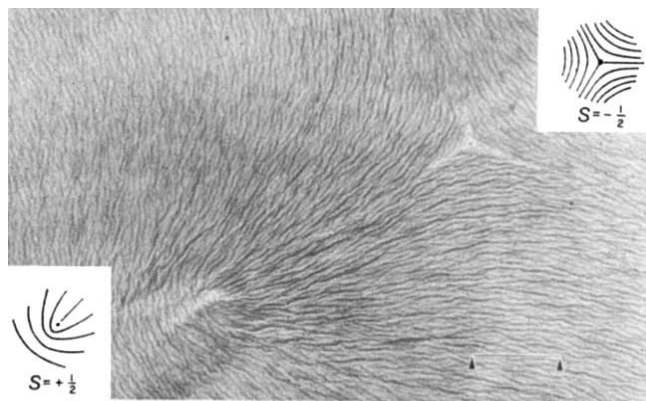


Fig. 2 TEM image of disclination dipole in a thermotropic liquid crystalline polymer and schematic (insets) of molecular director trajectories around a positive and a negative half-integer disclination. Scale bar, 10 μm .

to consist of an assembly of 'effective domains' due to a particular arrangement of disclinations. In two dimensions, Marrucci's scheme consists of a group of two positive and two negative half-integer disclinations (Fig. 1). This particular array yields an approximately zero average net director orientation over the basic domain and hence zero global sample orientation. Moreover, the molecular director changes continuously over the polydomain sample except at the disclination cores. Wissbrun⁶ has further developed Marrucci's model to allow the size (or number density) of the domains to change during flow, with consequent changes in the rheological characteristics. Recent flow visualization experiments using light microscopy have suggested that the flow can both increase⁷ and decrease⁸ the number of defects in the fluid. To date, no detailed experimental observations of the proposed disclination-domain structures have been made.

Recent transmission electron microscopy (TEM) studies of alternating rigid/flexible thermotropic LCPs conducted in our laboratory have permitted direct visualization of the molecular director pattern in flow-oriented thin films at a resolution unmatched by previous light micrographs⁹. The mesophase texture and defects existing in the polymer melt state are retained by thermal quenching of the polymer fluid to room temperature. By annealing the glassy liquid crystalline polymer film above its glass transition but below the melting point, crystalline lamellae grow perpendicular to the local chain axis and effectively 'decorate' the molecular director field throughout the sample.

Samples were prepared by annealing of substrate-free oriented thin films of the thermotropic polyester from 1,10-decane bis(terephthaloyl chloride) and hydroquinone^{9,10}. Figure 2 is a TEM image of a region containing two singularities in the field of oriented long, thin crystalline polymer lamellae. Electron diffraction proves that the chain axis is normal to the long

dimension of the lamellae. Thus, the set of lamellar normals represents the set of molecular director trajectories over the sample. As was recently pointed out by Frank¹¹, the lamellar decoration pattern demonstrates that the molecular director field consists of translationally parallel trajectories, that is, families of congruent curves such that the vector joining points of the same slope on any two members of the family is a constant. The trajectories of the lamellar normals in the vicinity of each defect corresponds quite well with that expected from the molecular director trajectories associated with 180° disclination lines running perpendicular to the specimen plane. The molecular director trajectories about such isolated defects are in good accord with those predicted nearly 30 years ago by Frank¹². Dipoles consisting of a positive and negative 180° disclination at a typical separation of micrometres are frequently observed. Apparently, annihilation occurs if pairs of opposite sign disclinations approach each other more closely. The core regions of the negative (threefold symmetric) disclination appear bright, which indicates a local thinning of the film, possibly corresponding to a high concentration of chain ends and hairpin molecular folds.

In addition to isolated and variously paired disclination dipoles, arrays involving approximately equal numbers of positive and negative disclinations are frequently observed. A diamond-shaped group involving two positive and two negative half-integer disclinations have been seen repeatedly (Fig. 3a). Details of the molecular director trajectories are shown in Fig. 3b. The molecular director pattern is nearly identical to that of the hypothetical "effective domain" structure envisaged by Marrucci (see Fig. 1). The groups of four disclinations observed also resemble the 'lozenges' defined by edge dislocations of layers in cholesteric small-molecule liquid crystals as described by Bouligand¹. Groups of two positive and two negative disclinations (although of integer strength) were first drawn by Lehmann in a 1918 monograph¹³.

A larger group of disclinations is shown in Fig. 4a. A several square micrometres region of uniform orientation is bounded by a serrated wall of 10 alternating positive and negative disclinations. Bouligand's¹ schematic of a serrated wall structure is shown in Fig. 4b. The discrete appearance of domains suggested by the images shown in Fig. 3a and 4a results from observing the field of normals to the molecular director trajectories rather than the trajectories themselves¹¹. Regions of approximately

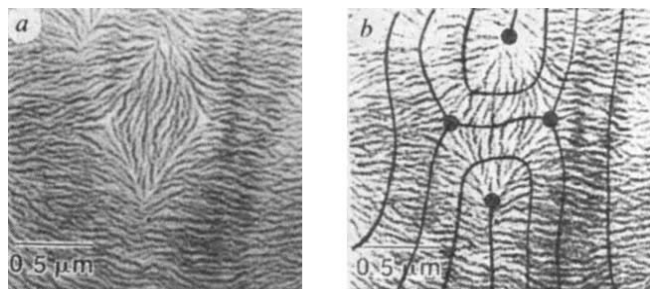


Fig. 3 a, TEM image and b, schematic overlay of lamellar trajectories in a diamond-shaped region defined by two pairs of oppositely signed half integer disclinations. Scale bars, 0.5 μm .

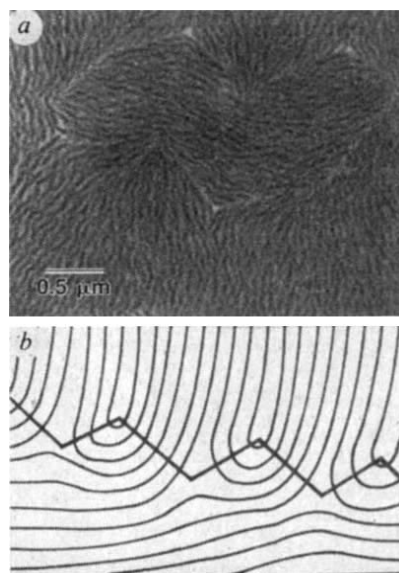


Fig. 4 a, TEM image of region of approximately uniform orientation defined by walls of alternating positive and negative disclinations and b, schematic of a disclination wall (ref. 1). Scale bar, 0.5 μm .

uniform orientation are enclosed by disclinations where continuous rotation of the molecular director occurs about an axis parallel to the disclination lines (Neel walls in the language of magnetism). Except in the vicinity of the disclination cores, the field of molecular director trajectories is both continuous and translationally parallel.

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Rapid reduction of nitrogen oxides in exhaust gas streams

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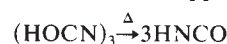
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Nitrogen oxides (NO_x) play a major role in the formation of photochemical smog and in acid rain production¹. Some progress has been made in reducing NO_x emissions through the use of combustion and exhaust control schemes, including three-way catalyst, staged combustion, and ammonia injection. Nevertheless, estimates indicate that the production of nitrogen oxides will continue to increase until the end of the century and beyond, if current trends continue². We describe here a new chemical process capable of completely removing NO_x from the products of combustion. The effectiveness of this process is established in flow tube experiments. Further, we demonstrate the practical feasibility of this method by eliminating NO_x from a portion of the exhaust from a single-cylinder diesel engine. Based on these results we conclude that this process for rapid reduction of nitrogen oxides could play a major role in controlling NO_x emissions from most combustion devices.

Nitrogen oxide formation in combustion has received much attention^{3–7}. The recognition⁴ that ammonia can be used to reduce nitric oxide in exhaust gases led to major efforts to

characterize reactions important in the Thermal De NO_x process. The mechanisms and models that resulted have greatly improved understanding of nitrogen chemistry in combustion. For example, this research led to the prediction of nitric oxide concentrations in ammonia injection schemes^{5,7} and in general nitrogen chemistry in combustion^{6,7}. Experimental efforts have continued to increase our understanding of the radical species important in the conversion of fuel-bound nitrogen to N_2 and NO during combustion. Perry⁸ studied reactions of the NCO radical with combustion species. NCO radicals formed from the photolysis of isocyanic acid (HNCO) react very rapidly with nitric oxide, suggesting⁸ that this reaction might be important in nitrous oxide formation in combustion. These observations, combined with previous insights into nitrogen chemistry, led to a new scheme for NO_x reduction⁹.

The proposed nitrogen oxide reduction scheme is based on the addition of isocyanic acid to an exhaust stream containing NO_x . Isocyanic acid is formed from the decomposition of cyanuric acid (a nontoxic, commercially available compound) when cyanuric acid is heated above approximately 330 °C



When HNCO is mixed with an exhaust gas stream at temperatures ≥ 400 °C, a series of reactions occur that result in the loss of HNCO and NO . Using a long-pass optical cell with Fourier transform infrared (FTIR) detection, carbon monoxide, carbon dioxide, water, and nitrogen (by inference) are observed as products. Table I shows results obtained in initial flow reactor experiments to study the overall chemistry of the proposed scheme. This process has been used to remove more than 99% of the nitric oxide emissions in simulated and real exhaust gas streams containing from 250–3,000 p.p.m. by volume of NO .

The flow reactor was a 3.8 cm diameter stainless steel tube 1 m long, enclosed in a furnace. The simulated exhaust gas was sampled with a 6.4 mm quartz probe; the position of the probe tip was varied over a range of 70 cm by the use of a stepping motor. The flow rates of the gases used ($\text{Ar}/\text{NO}/\text{HNCO}$) were controlled and measured with calibrated mass-flow controllers. Residence times in the flow reactor were determined from the equation $t = Ad/V$ where t is time, V is flow rate, A is the cross sectional area of the tube, and d is the distance from the point of mixing. The temperature of the tube was measured using chromel/alumel thermocouples, and the pressure in the tube was measured using a capacitance manometer.

Although the initiation chemistry is uncertain, some observations regarding the overall chemical process can be made from the flow-tube experiments. The most prominent feature of the results (Table 1) is the decrease in NO and HNCO concentration and the formation of CO and CO_2 . It is evident from the data that the process occurs on a 1:1 molar basis.

Table 1 Flow data obtained from stainless steel flow reactor using simulated exhaust gases ($\text{Ar}/\text{NO}/\text{HNCO}$ mixture)

Temperature (°C)	Pressure (torr)	$[\text{NO}]_i$ (p.p.m.)	$[\text{HNCO}]_i^*$ (p.p.m.)	$[\text{NO}]_f^\dagger$ (p.p.m.)	$[\text{CO}]^\ddagger$ (p.p.m.)	$[\text{CO}_2]^\ddagger$ (p.p.m.)	$[\text{HNCO}]_f^\ddagger$ (p.p.m.)	Time (s)
585	508	0	660	0	420	190	50	0.30
582	512	740	610	410	30	500	80	0.30
639	540	725	695	120	100	520	75	0.30
638	539	500	635	70	130	425	80	0.59
647	637	370	350	100	40	250	60	0.15
647	537	365	485	95	80	345	60	0.72
640	537	725	720	80	130	525	65	1.15
643	532	725	805	45	160	600	60	1.40
663	537	670	925	40	80	800	20	2.00
651	603	530	555	115	65	450	40	0.35
644	537	725	855	25	210	600	45	1.70
643	537	725	820	45	160	600	60	1.40

* $[\text{HNCO}]_i$ is the initial HNCO concentrations calculated assuming carbon mass balance from the equation $[\text{HNCO}]_i = [\text{HNCO}]_f + [\text{CO}_2] + [\text{CO}]$

$^\dagger [\text{NO}]$ determined by NO_x chemiluminescent analyser.

$^\ddagger [\text{CO}]$, $[\text{CO}_2]$ and $[\text{HNCO}]$ measured using absorption at selected lines precalibrated with known concentrations of CO , CO_2 , HNCO .

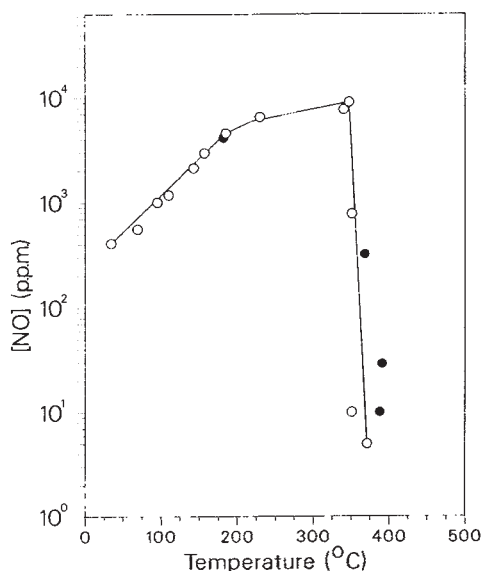
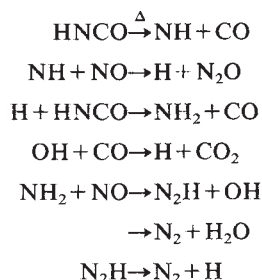


Fig. 1 Plot of NO concentration (p.p.m.) in treated portion (1.01 min^{-1}) of diesel engine exhaust gas versus cyanuric acid bed temperature ($^{\circ}\text{C}$). The diesel engine was operated at medium load (overall equivalence ratio of 0.5) and 1,800 r.p.m. Instrumentation sensitivity is limited to ~ 5 p.p.m. Data were obtained on successive days, with solid circles taken initially and open circles obtained in separate experiments.

The following possible reaction scheme, when combined with previously developed models⁵⁻⁷, may explain these experimental results:



This reaction scheme implies that, following initiation, the sequence through N_2H produces a growth of one hydrogen atom per cycle to sustain the chain. One important aspect of the chemistry of our proposed method is the absence of the need for controlled amounts of oxygen, suggesting that it could be readily implemented as a control process, if the presence of oxygen does not greatly change the mechanism.

To test the effect of excess oxygen on the process, and to develop a technology for exhaust conditions that are presently untreatable by existing technologies, such as 3-way catalyst and ammonia injection, we used the proposed method to reduce the nitrogen oxides in a portion of a diesel engine exhaust gas stream. The experiments used a single cylinder, 7.2 horse power Onan diesel engine. The engine was operated at a medium load condition (an overall equivalence ratio of 0.5) and an engine speed of 1800 r.p.m. A continuous sample of unfiltered exhaust gas was drawn at a rate of 1 l min^{-1} and passed over cyanuric acid in a 14.6 cm long by 4.72 cm diameter stainless steel tube with a volume of 256 cm^3 (the cyanuric acid bed). The sample then flowed through a 17.8 cm long by 2.2 cm diameter tube packed with two sizes of stainless steel spheres, diameter 0.55 cm and 0.64 cm (the packed bed), which had an open volume of 31 cm^3 and a total surface area including steel spheres of $1,630 \text{ cm}^2$. The packed bed was used to test the effect of surface-to-volume ratio and residence times on the chemistry. Finally, the exhaust gas flowed through a NO_x chemiluminescence

analyser. Samples also were taken of the treated and untreated exhaust gas for FTIR analysis.

Figure 1 shows the results of the diesel engine experiments. The NO concentration measured in the treated exhaust gas is plotted as a function of the cyanuric acid bed temperature. For cyanuric acid bed temperatures above 360°C the NO_x was essentially eliminated. These results were insensitive to packed bed temperatures between 450°C and 900°C , provided that the cyanuric acid bed temperature was greater than 360°C . At lower acid bed temperatures, nitric oxide levels in excess of that produced by the diesel engine (410 p.p.m.) were observed (Fig. 1). Surface interactions of HNCO with oxygen are believed to be important in this effect, and suggest the need to minimize the surface area in designing a practical device. Experiments using gaseous HNCO injection in oxidizing environments, which show that NO reduction takes place efficiently in the presence of excess oxygen, will be reported with related results in detail.

In summary, we have discovered a chemical means of removal of NO_x from the products of combustion or industrial emissions, and we have demonstrated its feasibility on a portion of the exhaust from a diesel engine. This process has important implications for a wide spectrum of energy conversion technologies and for industrial applications that presently produce NO_x as exhaust byproducts.

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A magmatic heat pump

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The structures of igneous cumulates can provide valuable clues to inaccessible magmatic processes. Many mafic layers in the Rhum Intrusion have 'finger structures'¹⁻³ that protrude into the overlying felsic rocks. These structures are attributable to melting of the overlying olivine-feldspar rocks by hot mafic magma emplaced as sills⁴. The bases of mafic layers contain smooth-bottomed olivine cumulates. Here I show that the heat needed to melt the roof of the layer is carried by rapidly rising light solute released by growth of basal olivine crystals that were deposited by two phase-convection. Compositional convection therefore drives a heat pump transferring heat of fusion from remote growing crystals to those that are melting. This action contrasts with cooling under a refractory roof, which is conduction-limited. The heat pumping principle shows why ultramafic magmas may melt their roofs whereas felsic magmas tend to form upper border zones.

Details of the crystallization sequence in the finger structures reported at Rhum have led to the realization that the mafic layers are pene-contemporaneous sills whose presence has caused melt-corrosion of the overlying rock⁴. Details are clarified

in ref. 4, but the features central to the present discussion may be summarized as follows. Finger structures occur only in troctolite (olivine-plagioclase rock, often called allivalite) overlying mafic layers rich in olivine cumulate. They are interpreted as melting features because the host rock fabric is continuous across the fingers, which therefore represent passive removal of felsic material and subsequent crystallization of mafic material. The crystallization sequence olivine-pyroxene in the fingers shows that the mafic material was supercooled when it finally solidified in the finger spaces⁴. The initial mafic magma was hot enough to dissolve the troctolite, so it was in the olivine field, not yet co-saturated with plagioclase feldspar and certainly not with pyroxene. The mafic layers have thicknesses commonly in the range 5–100 cm.

Melt features seem to be absent at the bases of the mafic layers. (There are load structures into crystal mushes of troctolite³, but these do not require melting at the local site.) The magma layers were emplaced into essentially solid troctolite; mineral compositions above and below the layers are similar, typically $\text{Fo}_{84\pm 2}$ in olivine, for example⁵. The initial state immediately after injection was therefore that of a hot, essentially isothermal magma layer sandwiched between cooler felsic rocks. Melting should have begun immediately at both boundaries. Instead, there is abundant evidence of melting at the upper boundary, but no firm evidence of melting at the lower.

The first obvious difference between the top and bottom boundaries is the presence of a basal olivine cumulate layer below (Fig. 1). This crystal layer can act as a thermal blanket and as a physical barrier to percolation of hot, dense liquid. Three kinds of convection are relevant: two-phase, compositional, and thermal. The rapid deposition of olivine crystals is promoted by two-phase convection^{6,7} of crystal-liquid suspensions from the cooler top of the mafic magma layer. (This result differs from those based on calculations of the Rayleigh number that suggest enduring turbulence and suspension instead⁸. However, the latter were reached without consideration of two-phase convection. An alternative route to a quite different set of Rayleigh numbers has been suggested by Marsh⁹.) Growth of olivine releases a light solute¹⁰ that convects freely upward¹¹; it also locks the local temperature onto a thermal maximum called a 'latent heat hump'¹². The resulting compositional convection is an important agent in causing solidification of the porous olivine cumulate^{10,11,13,14}. The rapid formation of such a solid caprock is required to prevent percolation of the magma to the underlying layer. The entire convective cycle is driven by absorption of heat at the roof of the mafic magma layer, which, in turn, drives two-phase convection, which carries downwards the heat sink needed to induce growth at the floor and thereby to induce compositional convection.

Thermal convection runs in the same direction as compositional convection but is less important. A useful measure of the relative potentials of compositional versus thermal convection is provided by the density ratio $R_p = \beta \Delta S / \alpha \Delta T$, where $\beta \Delta S$ is a compositional expansion times a concentration change and $\alpha \Delta T$ is a thermal expansion times a temperature change. The density ratio has been evaluated for the growth of cumulates¹⁵, and found to lie between six and 10^6 , with the larger values more common. Such large values show that the compositional contribution to buoyancy greatly exceeds the thermal contribution, and hence that the convection is dominantly compositional.

Corrosion of the roof rocks is extensive (Fig. 1). The latent heat released by the crystallization of 1 g of olivine can melt 2 g of troctolite¹⁶. Accounting for the lower density of the troctolite, a given volume of dunite can account for removal of ~2.5 times as much troctolite. The olivine present in the rest of the mafic layer, along with the basal olivine, can easily account for the missing troctolite in terms of latent heat. The thermal budget of a crystallizing magma sheet melting its roof was addressed by Irvine¹⁷. Applying the relations of his Fig. 14 to the present case, it can be judged that about twice as much cumulate can

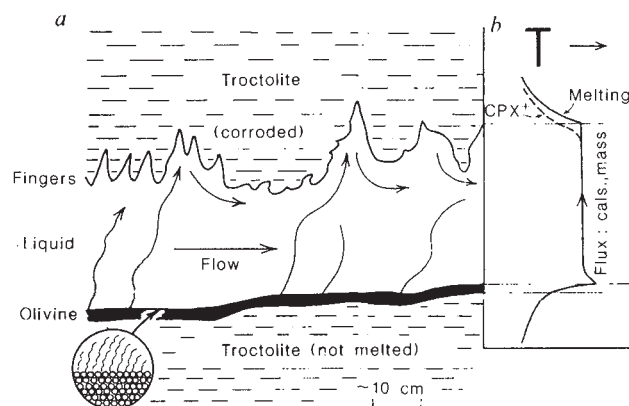


Fig. 1 *a*, Rhum finger structures above a mafic layer with an olivine cumulate base (black), after a photograph (Fig. 163 of ref. 2). The cotectic felsic+mafic material (troctolite) below the mafic layer shows no evidence of melting, in contrast to that in the base of the overlying layer. This asymmetry is accounted for by strong compositional convection of light rejected solute away from the growing basal olivine layer (shown by the magnified view in the lower circle). This convection pumps the heat of fusion through the mafic liquid to melt the overlying troctolite, where heat is absorbed. The thermal relations are shown schematically in *b*. When the convectational stirring becomes weak, supercooling within the fingers allows metastable growth of pyroxene (dashed curve labelled CPX^+). The thermal maximum (latent-heat pump) at the top of the olivine cumulate arises from the local maximum of latent heat released¹². A small supercooling of the average magma is assumed, to allow for nucleation of olivine. The mafic magma may be 100 °C or so hotter than the troctolite.

form when the heat evolved is absorbed by melting at the roof, compared to conduction alone through the roof.

It is proposed, therefore, that recrystallization of olivine in the mafic magma released most of the heat needed to dissolve the roof rocks. Rising streams of rejected solute (Fig. 1) will stir the magma layer and carry heat of crystallization away from the growing interface¹². Complementary two-phase plumes of olivine-seeded magma will carry supercooling downwards to allow growth of the basal cumulate, which is aided by conduction through the floor. The resulting temperature profile is portrayed in Fig. 1. The latent-heat hump occurs where the most olivine is growing, near the cumulate interface. The average magma temperature is shown as supercooled to allow for the nucleation and growth of olivine and eventual metastable crystallization of pyroxene. Physical stirring by the rising rejected solute keeps the liquid essentially isothermal. The ambient temperature at the roof is high enough to melt the roof rocks. The compositional convection therefore acts as a heat pump from the crystallizing base of the layer to the melting roof. The melted material is flushed away from the propagating fingers and dissolved in the feldspar-undersaturated mafic magma. The process must be rapid to avoid heating the walls of the fingers excessively, because they ultimately chill the liquid into the metastable pyroxene field⁴. The chilling presumably occurs when the heat-pumping process has died down below some critical threshold velocity.

The magmatic heat pump is similar to a classical isothermal heat pipe driven by the counterflow of two phases such as water and steam. The magma layer is also sensibly isothermal, but here the counterflow consists of a two-phase flow originating at one interface balanced by a single-phase, density contrast flow originating at the other. The pump and pipe are similar in transferring latent heat from one boundary to another.

Melting on a much grander scale evidently occurred at the Muskox Intrusion in Canada, where the roof rocks of the intrusion were extensively melted¹⁷ during the crystallization of the olivine-rich cumulates. It seems that heat pumping by

compositional convection occurred here on a massive scale. The paired heat source (olivine cumulate) and sink (fusible roof rocks) were both present, and compositional convection must have operated strongly. The pumping action could be damped but not erased by any convective stirring, because compositional convection will act over at least part of the distance of travel. If paired two-phase and compositional convective streamers or plumes dominated the convective flow pattern, heat pumping may have acted directly from floor to roof.

By contrast, felsic cumulates have a dense rejected solute¹⁰ that cannot transport heat upwards through the overlying magma. Roof melting would therefore not be expected in felsic intrusions and frozen upper border zones are observed at such felsic intrusions as Skaergaard¹⁵ and Kiglapait¹⁰. The rollover point between dense and buoyant rejected solute occurs, in mafic magma series, approximately at magnetite saturation¹⁵, hence magnetite-bearing calc-alkaline magmas can more readily melt their roofs by heat pumping from cumulates than tholeiitic and more reduced magmas. This principle accords with the common impression that calc-alkaline series magmas tend to show more contamination effects than Fenner-trend magmas. The extended calc-alkaline effect is much weaker than the effect seen in very mafic magmas, because the latent heat of fusion of magnetite is less than that of magnesian olivine and magnetite generally crystallizes in company with feldspar, so that the bulk

contributions of both latent heat and buoyant solute are small compared with magmas crystallizing olivine.

Although roof melting is not anticipated for felsic intrusions, the drainage of hot, dense solute from felsic roof cumulates implies that floor melting might occur by heat pumping. This could be the case for certain anorthosites and other felsic cumulates early in their history. However, this process is self-limiting because it produces an upper thermal blanket of roof cumulates through which conduction will have to occur for the magma to crystallize. By contrast, heat pumping with roof melting continually exposes cooler material to the magma, thus maximizes cooling and crystallization rates¹⁷.

Heat pumping by compositional convection may account for roof melting on scales ranging from individual layers at Rhum to whole intrusions emplaced into fusible crust. Pumping is most efficient when the basal cumulate is ultramafic and the roof is readily melted. This combination may occur most commonly for early olivine cumulates, but might also occur for later magnetite cumulates. The crystallization of magnetite in company with other mafic crystals and feldspar renders most calc-alkaline cumulates weakly capable of pumping. Inverted heat pumps from felsic roof rocks are inherently self-limiting because they impose an insulating cumulate in the path of heat removal to the Earth's surface.

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Formaldehyde oxime leaching of metals from deep-sea manganese nodules and other ores

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Formaldehyde oxime, $\text{CH}_2=\text{NOH}$, has for a long time been recognized as a sensitive colorimetric reagent for manganese, copper and metals of the iron group. In alkaline medium, the oxime dissociates, forming the $\text{CH}_2=\text{NO}^-$ anion which bonds to multivalent metals, giving complexes of the general formula $\text{M}(\text{CH}_2\text{NO})_n^{(6-n)-}$, where n is the oxidation state of the metal^{1,2}. Here I report the results of experiments which show that aqueous formaldehyde oxime constitutes a new and effective leaching agent for several types of ores, such as pyrolusite, malachite, heterogenite and deep-sea manganese nodules. The reagent seems particularly attractive for nodules, as the leaching is rapid, in acid as well as in neutral and/or alkaline media, and at ambient and lower temperatures. Manganese nodules constitute an important potential source of nickel and especially cobalt: the amount of cobalt recoverable from the north-west equatorial Pacific alone is estimated³ to be 4×10^6 tonne—of the same order as the world total land-based reserves.

The formaldehyde oxime solutions were prepared by mixing solid hydroxylamine hydrochloride with an equimolar amount of a 38 wt% aqueous solution of formaldehyde. The reaction is immediate, yielding a concentrated solution of formaldehyde

oxime, which is then diluted and brought to the desired pH by addition of ammonia or sodium hydroxide.

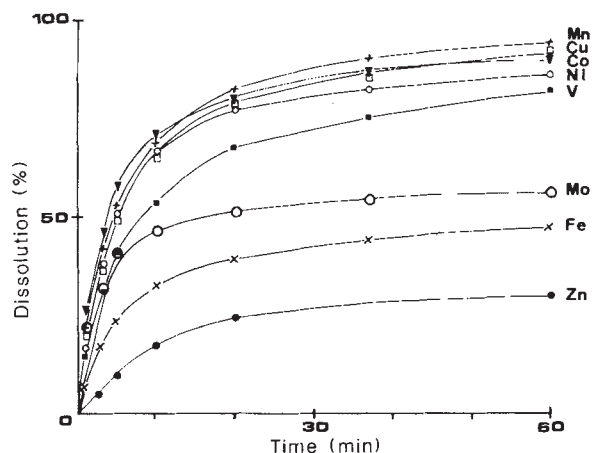
The exact origin of the nodules, collected during the 1974-78 campaign of Ocean Mining Associates, is unknown, but their composition (Mn 28.9, Fe 5.02, Ni 1.29, Cu 1.04, Co 0.24, Zn 0.13, Mo 0.065, V 0.051 wt% on dry basis) is very similar to that of the nodules from the Clarion-Clipperton zone (eastern Pacific Ocean)². The X-ray diffraction spectrum (Fe $K\alpha$ line) consisted only of the four major, broadened, lines of todorokite, plus the main quartz line.

Other materials selected for the leaching experiments were a phosphorus-rich manganese ore (Mn 31.6, Ca 13.0, P 0.25 wt %) from Raouisse (Vanuatu) in which we identified pyrolusite ($\beta\text{-MnO}_2$) and calcite, and a complex Zairian copper-cobalt ore, consisting mainly of badly crystallized heterogenite (CoOOH) and malachite, together with some muscovite and diasporite.

All these materials were ground to $<106 \mu\text{m}$. The dissolution experiments were carried out in a thermostated vessel, fitted with a simple magnetic stirrer. At intervals, samples were taken from the reaction medium, filtered and then analysed by atomic absorption or atomic emission (DCP) spectrophotometry.

Pyrolusite (as well as laboratory-grade MnO_2) dissolves easily in formaldehyde oxime solutions. From pH 5-11 (experiments beyond this limit were avoided as the oxime is sensitive to oxidation in alkaline medium) a brownish-red coloration appears instantaneously, evidence of the formation of the $\text{Mn}(\text{CH}_2\text{NO})_2^{2-}$ anion. The reaction is rapid: for example, at 20 °C and pH 9.8, with 1.5 g ore in 300 ml 1M formaldehyde oxime, >90% of the Mn passed into solution after 5 min, the leaching being complete after ~10 min. Under these conditions, the gangue minerals (calcite and apatite) were identified in the residue) were left practically unaltered.

Fig. 1 The extraction of various elements from eastern Pacific manganese nodules (for conditions see text).



Separate dissolution experiments made under the same conditions as above have shown that among calcite, dolomite, magnesite, fluoroapatite and goethite, which are main gangue minerals of manganese ores, only calcite dissolved to a significant extent (25 mg Ca per litre solution after 60 min).

The quantity of manganese dioxide that can be dissolved in formaldehyde oxime solutions corresponds roughly to the stoichiometry of the reaction, that is, the formation of the hexacoordinated complex. Thus 1 litre of 1 M formaldehyde oxime solution dissolves 0.14 mol MnO_2 (12 g) at 20 °C and pH 9.8.

When the dissolution is done in an acidic medium ($\text{pH} < 5$), the oxime is oxidized by MnO_2 , and colourless solutions of Mn(II) are produced.

The powdered nodules dissolve even faster than pyrolusite in formaldehyde oxime; the solutions are also very dark. To slow the reaction rate the dissolution experiments were done in an ice bath.

Figure 1 shows an example of the results. The conditions were: temperature 0 °C, pH 7.5, 1.5 g nodule in 500 ml 0.5 M formaldehyde oxime. The percentage of every element passing into solution is represented as a function of time.

Other experiments were made under different pH conditions: as a rule, the dissolution paths of Mn, Ni, Cu and Co are always very similar (these elements are closely associated in the todorokite structure) and are not influenced by the alkalinity of the medium, in the pH range ~6–11.

The amount of iron dissolved never exceeded 70% of the iron content of the nodules, even if the leaching experiment was prolonged to 24 h at ordinary temperature. The mineralogical support of this insoluble iron was not identified, as only quartz, labradorite—a feldspar-type mineral—and traces of a 7-Å clay mineral were identified by X-ray diffraction in the dissolution residue.

The dissolution behaviour of the other valuable elements of the nodules is more or less pH-dependent.

By changing the experimental conditions, and particularly by raising the nodule:oxime ratio, we leached most (75–95%) of the nickel, copper and cobalt, leaving 45% of the manganese and >99% of the iron in the solid residue. These results could have a long-term economic interest, and will be discussed later (R.D., in preparation).

The third substance studied was the complex copper-cobalt ore. We observed that heterogenite always dissolves faster than malachite, and to ensure complete extraction of copper in a reasonable time it is necessary to operate at ≥ 35 °C. We did not succeed in leaching copper or cobalt selectively. As an example, table 1 gives the percentages of these elements dissolved, as a function of time at 20 °C and pH 7.0, with 1.5 g ore in 500 ml 0.5 M formaldehyde oxime.

Because most of the oxide, hydroxide and carbonate ores of copper and cobalt are easily leached by ammonia and/or mineral acids, economic interest in leaching by formaldehyde oxime seems unlikely. It is nevertheless remarkable that this leaching may be done in a neutral medium.

Little is known about the formation constants of metal-formaldehyde-oxime complexes. The only published data are those of Bečka and Jokl⁴; the values are high ($\log K_{\text{form}} = 7.5, 9.8, 17.3$, and 18.9 respectively for the Cu (II), Mn (IV), Fe (III) and Ni (IV) complexes), indicating a stability much greater than, say, that of the ammine complexes of Cu and Ni. This alone cannot account, however, for the reactivity of formaldehyde oxime solutions towards the particular minerals studied here, as indicated by the fact that manganese dioxide is easily attacked by the oxime, but not iron oxyhydroxide (goethite), whereas the stability of the Fe (III) complex is seven orders of magnitude greater than that of the Mn (IV) complex.

The imperfections in the crystal lattices of the minerals must play a prominent role in their reactivity; this is probably also true for deep-sea manganese nodules, which have a poorly crystallized structure.

This reaction between nodules and aqueous formaldehyde oxime seems promising, as a rapid and more or less selective extraction of the valuable elements is attainable, even at low temperature. So far, however, only the leaching operation has been studied in our laboratory, and attention will be given to the recovery of the dissolved metals and the recycling of the oxime.

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Table 1 Dissolution kinetics of copper and cobalt from a complex heterogenite—malachite ore (for conditions see text).

Time (min)	Co dissolved, wt %	Cu dissolved, wt %
1	12	5
3	27	9
5	43	12
10	64	16
20	70	21
30	77	27
75	94	34
120	98	40

Homoeobox gene expression in mouse embryos varies with position by the primitive streak stage

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Pattern formation in animal development requires that genes be expressed differentially according to position in the sheets of cells that make up the early embryo. The homoeobox-containing genes of *Drosophila* are control genes active both in the establishment of a segmentation pattern^{1,2} and in the specification of segment identity^{2,3}. *In situ* hybridization experiments⁴⁻⁶ confirm that these genes are expressed in a segmentally-restricted manner and that their expression presages morphological differentiation of segmental structures. Homoeobox genes have recently been isolated from the mouse⁷⁻¹² and have been shown to be expressed during mouse development⁹⁻¹³. Using *in situ* hybridization, we show here that expression of the mouse homoeobox gene *Mo-10* (ref. 7) is spatially restricted in the developing embryo and that localization of expression is already evident within the germ layers before their morphological differentiation. These findings support the suggestion¹⁴ that the homoeobox genes of mammals, like those of *Drosophila*, may be important in pattern formation.

To examine expression of the *Mo-10* gene (also known as *Hox-1*, ref. 9), the 660-bp *HindIII-EcoRI* fragment containing the homoeobox was subcloned into pSP65 and used to produce high specific activity radioactive RNA probes complementary to *Mo-10* messenger RNA (Fig. 1). Two separate probes were made. Probe 1 contains 144 nucleotides from the homoeobox and ~70 nucleotides of additional 3' sequence. Probe 2 resembles probe 1, but includes the whole of the homoeobox plus ~400 nucleotides of 5' sequence. Both probes gave similar results in the *in situ* hybridization experiments.

The 9½ day-old embryo was the most advanced stage to be examined. By this age most of the organ systems of the body are already laid down (Fig. 2a). The most intense labelling is seen in the nervous system (Fig. 2b). Anterior parts of the neural tube, developing at this stage as the forebrain and midbrain regions are not labelled above background, but intense labelling begins posterior to a point in the hindbrain region just anterior to the otic vesicle, at the level of the second (hyoid) visceral arch. The whole length of the neural tube posterior to this point is labelled, but the concentration of grains declines in more posterior regions. We examined the boundary between labelled and unlabelled parts by phase contrast optics, but found no corresponding morphological change in the neural tissue. At this stage of development metameric segmentation of the hindbrain is apparent in its serial constrictions to form neuromeres. The boundary between labelled and unlabelled neural tissue coincides with the boundary between two neuromeres.

The pattern of labelling observed in non-neural tissue of the 9½ d embryo was less remarkable. Generally, labelling was above background in most posterior mesodermal structures, such as the mesonephric kidney and the somite blocks (not shown), but the labelling was weak compared to that of the nervous system. Mesoderm-derived tissues in the head (Fig. 2b), the heart, the liver, the gut, and the appendages all showed only background labelling.

At 7½ d of gestation, the cells that will form the mouse are arranged as the three germ layers: ectoderm, mesoderm and endoderm (Fig. 3a). But in the posterior region (the site of the

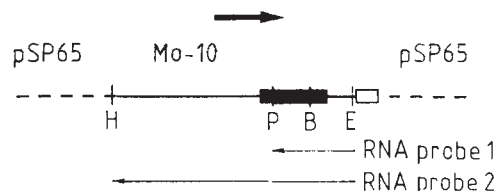


Fig. 1 Use of a pSP65 plasmid¹⁵ containing the *Mo-10* homoeobox to prepare radioactive RNA probes for *in situ* hybridization and Northern blotting. H, *HindIII*; P, *PvuII*; B, *BglII*; E, *EcoRI*. The homoeobox and the SP6 promoter are shown as filled and open boxes respectively. The normal direction of transcription of the *Mo-10* DNA is from left to right (bold arrow) based upon the conserved open reading frame⁷ and homology with the known direction of transcription of other homoeobox genes¹⁰⁻¹².

Methods. To construct the plasmid, the *HindIII-EcoRI* fragment containing the homoeobox was obtained from a cosmid clone of mouse genomic DNA¹⁶. The nucleotide sequence (not shown) of this homoeobox is more than 99% homologous to the published sequence⁷. Radioactive RNA probes complementary to putative *Mo-10* mRNA were prepared after digestion of plasmid DNA with either *PvuII* (probe 1) or *HindIII* (probe 2), followed by *in vitro* transcription using SPS6 RNA polymerase (Promega Biotec). Labelled ribonucleotide triphosphates used were: for *in situ* hybridization, ³H-UTP and ³H-CTP (Amersham; 20 µCi of each, carrier-free, per 20 µl reaction); for Northern blotting, ³²P-UTP (Amersham; 50 µCi, 0.2 µg per 20 µl reaction). Final specific activities were about 9 × 10⁷ d.p.m. per µg for ³H-labelled probes and 2 × 10⁸ d.p.m. per µg for ³²P-labelled probes. Probes for *in situ* hybridization were subjected to limited alkaline hydrolysis as described¹⁷ for either 45 min (probe 1) or 90 min (probe 2). Probes were then checked by gel electrophoresis to ensure that the bulk of the RNA was reduced to 50–150 nucleotides. A control probe of opposite sense to the probes described above was prepared after subcloning the *HindIII-EcoRI* fragment of *Mo-10* DNA into pGEM-4 (Promega Biotec), then using T7 RNA polymerase in the transcription reaction. This probe, hydrolysed as above, did not produce specific labelling when used in the *in situ* hybridization experiments.

primitive streak) ectoderm and mesoderm layers remain unseparated. By 7½ d (Fig. 3c), anterior ectoderm in the region of the head fold has become thickened and elevated, presaging the formation of the brain. *In situ* hybridization experiments on embryos at these stages show that labelling is restricted to posterior regions, including the primitive streak (Fig. 3b) and adjacent regions of ectoderm and mesoderm (Fig. 3d). The anteroposterior boundary between unlabelled and labelled regions is not sharply defined at these stages of development. From examination of twelve 7½ d to 7¼ d embryos cut in sagittal section, it was found that labelling varied from about one-third to two-thirds of the length of the embryo, with older embryos tending to show the greater extent of labelling. The extraembryonic tissues at these stages showed only background labelling.

In Northern blot analysis of poly(A)⁺ RNA extracted from 9½ d and 10½ d whole mouse embryos, probe 1 hybridized with two distinct transcripts of 4 kilobases (kb) and 1.7 kb (Fig. 4). The bands were detected after washing the filter under highly stringent conditions, suggesting that both transcripts are products of the *Mo-10* gene.

The *in situ* hybridization results are consistent with the hypothesis¹⁴ that the homoeobox genes of mammals, like those of *Drosophila*, may be important in the specification of body pattern. The two main observations which suggest such a role for the *Mo-10* gene are, first, that its expression in embryos is spatially restricted in tissues that appear uniform in cell composition on the basis of morphology alone and second, that spatial restriction in expression is evident very early in development, before any observable signs that the body pattern has already been laid down. Since precise fate maps are not available for mouse embryos, it is not possible to conclude that the labelled

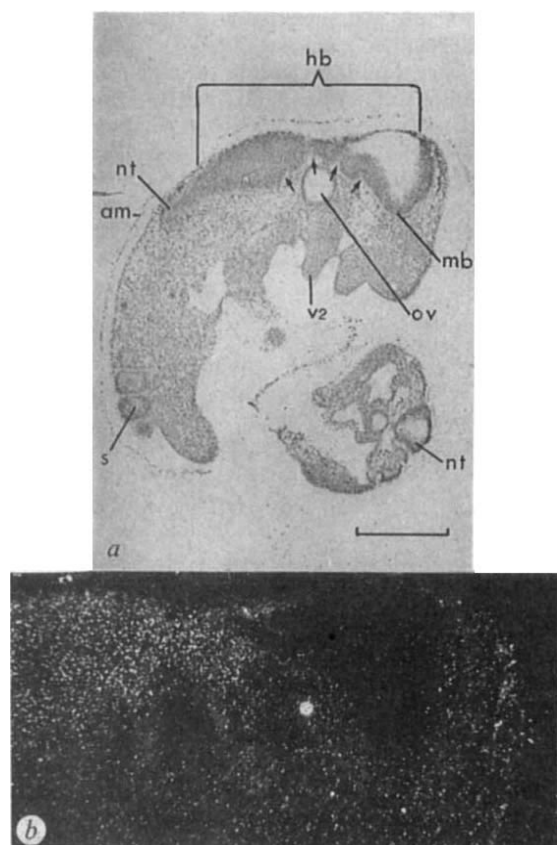


Fig. 2 Localization of *Mo-10* transcripts, using probe 2 (Fig. 1), by *in situ* hybridization on sections of the 9½ d mouse embryo. *a*, Embryo cut in parasagittal section, under bright-field illumination. hb, hindbrain; mb, midbrain; nt, neural tube; ov, otic vesicle; v2, second visceral arch; s, somite; am, amnion; arrows, constrictions between neuromeres. Scale bar, 0.5 mm. *b*, Silver grains in the head region of the same section, viewed under dark-field illumination. The most intense labelling was detected in the nervous system, but labelling was not seen in anterior regions of the brain. The boundary between labelled and unlabelled neural tissue was located in the hindbrain at the junction of two neuromeres.

Methods. Embryos were obtained from natural matings between F₁ (CBA × C57Bl/6) mice. The morning that the copulation plug was found in the vagina was designated day ½ of pregnancy. After dissection from the uterus into phosphate buffered saline, the embryos were fixed in Bouin's fluid for 24 h at 4°C. They were then dehydrated in ethanol, cleared in xylene, and embedded in paraffin wax (m.p. 56°C). Sections (10 µm thick) were mounted on poly-L-lysine treated glass microscope slides, dried at 37°C for 4 h, de-waxed in xylene, rehydrated in decreasing ethanol concentrations and digested with proteinase K as described¹⁷. Treatment with acetic anhydride and the conditions of hybridization, digestion with RNAase A and washing were as described¹⁸. After the final wash, sections were immersed in 0.3 M ammonium acetate in 95% ethanol and allowed to dry in air. Slides were dipped at 50°C in Ilford L4 emulsion diluted 1:2 with water, allowed to dry and stored at 4°C. After an exposure time of three weeks, the autoradiograms were developed in Kodak D19 developer for 3 min at 20°C. The tissue sections were then stained in Giemsa, dehydrated in ethanol and mounted under coverslips in DPX mountant.

ectoderm tissue seen in the 7½ d embryo would normally have given rise exactly to the labelled neural tissue seen at 9½ d. Before drawing such a conclusion it would be necessary to examine several intermediate stages since the primitive streak region is one of considerable cell proliferation and migration.

It is possible that the spatial restriction of gene expression

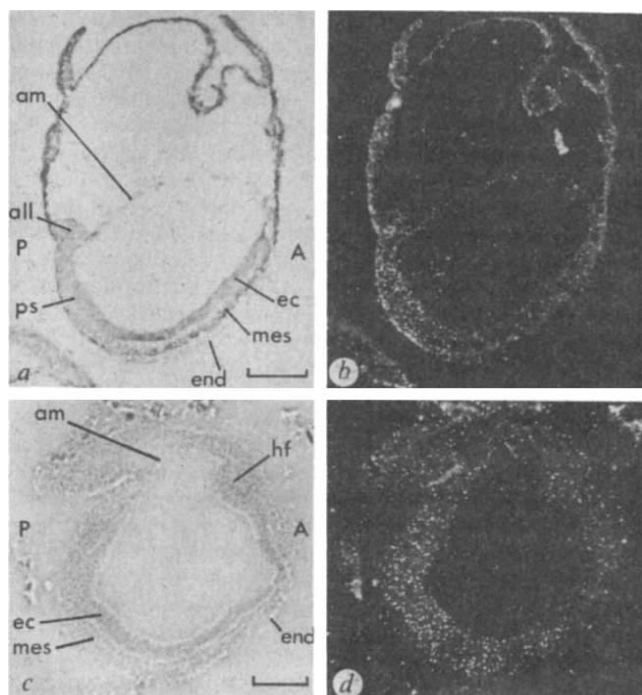


Fig. 3 *In situ* hybridization to detect *Mo-10* transcripts in sections of primitive streak stage mouse embryos. *a, b*, 7½ d embryo in parasagittal section examined with probe 2; *c, d*, 7½ d embryo in parasagittal section, probe 1. *a* and *c* show sections by bright-field and phase contrast illumination respectively. A, anterior; P, posterior; ps, primitive streak; ec, ectoderm; mes, mesoderm; end, endoderm; am, amnion; all, allantois; hf, head fold. Scale bars, 0.1 mm. *b* and *d* show the sections by dark-field illumination. Labelling was restricted to the posterior regions of the embryonic tissue, including the primitive streak (*b*), ectoderm and mesoderm (*d*). The bright border seen at some positions along the edge of the embryonic and extraembryonic tissues in *b* is not due to silver grains, but to a light scattering effect by material which was often present at this stage. Methods were as for Fig. 2.

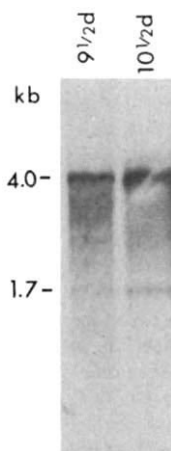


Fig. 4 Northern blot hybridization of *Mo-10* probe 1 (Fig. 1) to poly(A)⁺ RNA isolated from whole mouse embryos of 9½ and 10½ d gestation. **Methods.** RNA was prepared as described¹⁹. About 5 µg of each poly (A)⁺ RNA sample was separated by electrophoresis through a formaldehyde-agarose gel. RNA was then blotted on to Gene Screen Plus. The filter was prehybridized for 2 h at 60°C in 1 M NaCl, 50 mM Tris HCl pH 8.0, 1% SDS, with 100 µg ml⁻¹ *Escherichia coli* transfer RNA and 10 µg per ml sonicated salmon sperm DNA. Hybridization with ³²P-labelled probe 1 was carried out overnight under the same conditions but with 10% dextran sulphate added. The concentration of probe was about 2 ng ml⁻¹. The filter was washed twice in 2 × SSC, 1% SDS at 65°C for 20 min, then once in 0.1 × SSC, 1% SDS at 65°C for 20 min before autoradiography.

that we have described is not primarily concerned with pattern formation, but instead reflects some other developmental difference between the trunk and head regions of the mouse. However, some observations recently described for the mouse homoeobox gene *Mo-EA*¹¹² suggest further parallels between patterns of expression of homoeobox genes in *Drosophila* and

in mice. Awgulewitsch *et al.*¹² used *in situ* hybridization to localize *Mo-EA* transcripts in the nervous system of newborn mice. Labelling was absent in anterior regions of the nervous system but began at about mid-cervical region. Labelling was most concentrated at this point, becoming less intense in more posterior regions of the spinal cord. Thus, *Mo-EA* transcripts, like *Mo-10* transcripts, are detected only posterior to a discrete boundary in the nervous system. This boundary is in the mid-cervical region for *Mo-EA* and in the hindbrain region for *Mo-10*. It should be remembered that transcription of these two genes was examined at different stages in the development of the mouse, but the apparent distinction in the sites of transcription between the two genes is reminiscent of the localized expression of *Drosophila* homoeobox genes within the ventral nerve cord of the developing fly^{3,6,20}.

We cannot rule out the possibility that the minor 1.7 kb transcript detected in our Northern blots might be due to cross-hybridization of the *Mo-10* probe with a product of another homoeobox gene. But we think it is more likely that the two transcripts result from differential processing of a single gene, as suggested for other mouse homoeobox genes^{9,13}. Variability in transcript size could then result from use of alternative initiation, termination, or splice sites. The transcription patterns of the *Mo-10* gene will become amenable to more detailed analysis when complementary DNA probes become available. We consider it unlikely that our *in situ* hybridization experiments could detect any transcripts not shown on the Northern blots since the conditions of hybridization used *in situ* were more stringent than those used in Northern blotting²¹.

The potential of the *Mo-10* gene for a role in pattern formation

is clear. For example, gene expression at an abrupt boundary within the hindbrain might serve as a cue, instructing cells immediately posterior to this point to develop differently from those cells anterior to the boundary. Such a mechanism would be similar to that proposed for homoeobox genes in the bithorax complex of *Drosophila*^{3,22}. From our findings, it is apparent that *Mo-10* gene expression could serve as a positional cue in this way in both ectoderm and mesoderm-derived structures.

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Differential and stage-related expression in embryonic tissues of a new human homoeobox gene

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The homoeobox is a 183 base-pair (bp) DNA sequence¹ conserved in several *Drosophila* genes controlling segmentation and segment identity²⁻⁴. Homoeobox sequences have been detected in the genome of species ranging from insects and anellids to vertebrates^{5,6} and homoeobox containing genes have been cloned from *Xenopus*⁷⁻⁹, mouse¹⁰⁻²² and man²³. We recently isolated human homoeobox containing complementary DNA clones, that represent transcripts from four different human genes²⁴. One clone (HHO.c10) is selectively expressed in a 2.1 kilobase (kb) polyadenylated transcript in the spinal cord of human embryos and fetuses 5-10 weeks after fertilization²⁵. We report the characterization of a second cDNA clone, termed HHO.c13, that represents a new homoeobox gene. This clone encodes a protein of 255 amino-acid residues, which includes a pentapeptide, upstream of the homoeo domain, conserved in other *Drosophila*, *Xenopus*,

murine and human homoeobox genes. By Northern analysis HHO.c13 detects multiple embryonic transcripts, which are differentially expressed in spinal cord, brain, backbone rudiments, limb buds and heart in 5-9-week-old human embryos and fetuses, in a striking organ- and stage-specific pattern. These observations suggest that in early mammalian development homoeobox genes may exert a wide spectrum of control functions in a variety of organs and body parts, in addition to the spinal cord.

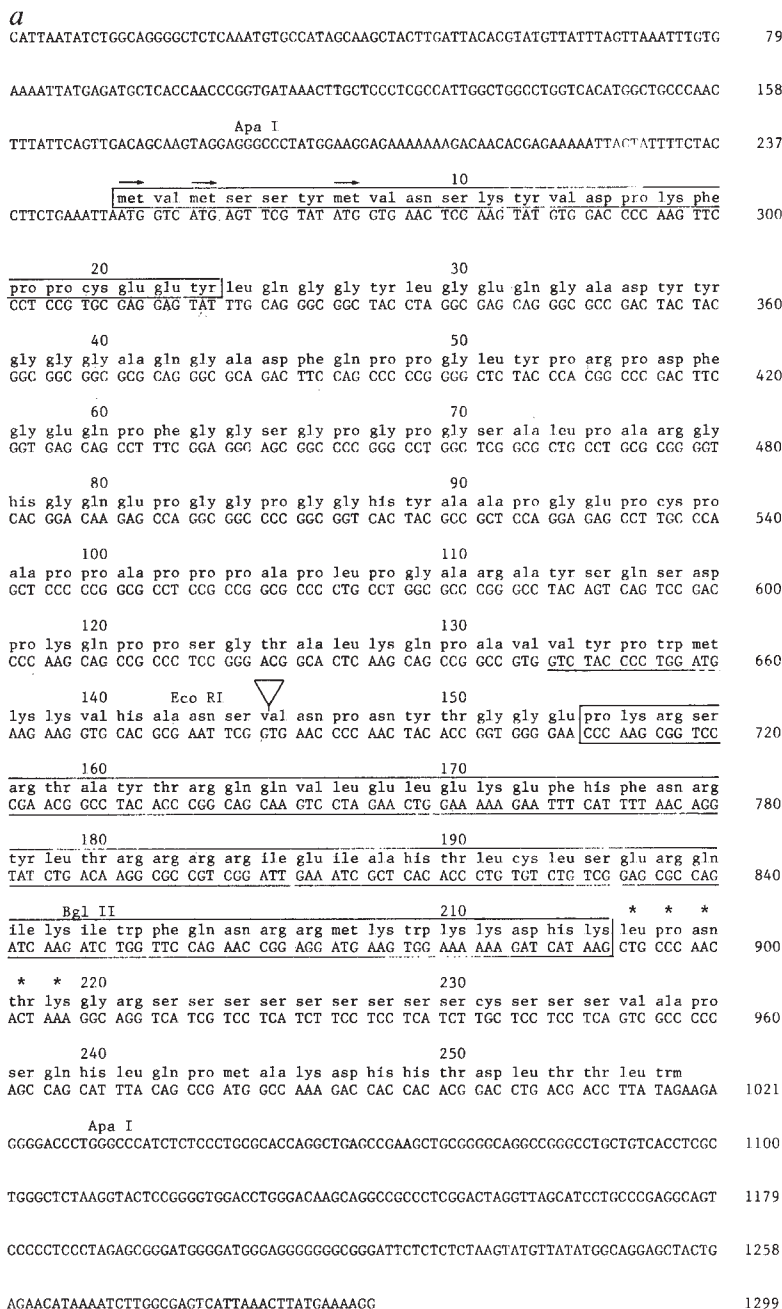
The nucleotide sequence of clone HHO.c13 is shown in Fig. 1a. Multiple stop codons are present in each frame up to nucleotide 230. Translation of the longest reading frame from the ATG codon at base 250 would produce a peptide sequence of 255 amino-acid residues, containing a homoeo domain extending up to 41 residues upstream from the carboxy terminus. The putative 285-bp 3'-untranslated (3'-UT) region includes a polyadenylation signal (AUUAAA) 18 nucleotides upstream from the poly(A) tail. As shown in Table I, the sequence of HHO.c13 homoeobox is very similar (~60-75% nucleotide homology) to that of other homoeoboxes. At the level of protein sequence, the HHO.c13 homoeo domain shows particularly good homology with murine *Hox1-3* (98.3%), *Xenopus Xhox-1A* (93.4%), human HHO.c10 (90.1%) and *Drosophila Deformed* (*Dfd*) (90.2%) domain. Interestingly, the pentapeptide Leu-Pro-Asn-Thr-Lys immediately downstream from the HHO.c13 homoeo domain (Fig. 1a) is conserved in the *Dfd*^{26,27}, *Xhox-1A*⁹ and *Hox1-3*²⁰⁻²² genes.

The putative protein encoded by HHO.c13 contains the pentapeptide Val-Tyr-Pro-Trp-Met, starting at amino-acid position 133 (-21 from the homoeo domain). As shown in Fig. 1b, this sequence is very similar to the Ile-Tyr-Pro-Trp-Met peptide in two other human unrelated cDNA clones containing homoeobox sequences, termed HHO.c1 and c8 (ref. 24). Its presence in HHO.c10 (ref. 25) cannot be assessed, because this clone is truncated within the homoeobox. The pentapeptide in HHO.c13 is conserved in *Xenopus Xhox-1A* gene⁹, whereas the pentapeptide in HHO.c1 and c8 is conserved in *Drosophila Dfd*²⁶, and mouse m6 (P. Gruss and M. Kessel, personal communication) genes. A highly homologous Leu-Tyr-Pro-Trp-Met

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Fig. 1 a, Nucleotide sequence of the human cDNA clone *HHO.c13*, with the theoretical translation of its longest open reading frame. Relevant restriction endonuclease recognition sites are also shown. Arrows, three methionine codons, the putative alternative translation start sites: none of them has very good homology with the consensus sequence of initiation sites in eukaryotic genes³⁷. The boxed N-terminal amino-acid sequence shows a high degree of homology with the corresponding peptide sequence of *Xenopus Xhox-1A*: Met-Arg-Met-Ser-Ser-Phe-Leu-Ile-Ser-Ser-Asn-Tyr-Val-Asp-Pro-Lys-Phe-Pro-Cys-Glu-Glu-Tyr (see ref. 9). The sequence encoding the homoeo domain is boxed. The pentapeptide Val-Tyr-Pro-Trp-Met, homologous to that found in a corresponding region of a variety of other homoeobox genes (see Fig. 1b) and the polyadenylation signal (ATTAAG)³⁸ preceding the poly(A) tail are underlined. Asterisks, the conserved pentapeptide downstream from the homoeo domain. ▽, position of the 541-bp intron. **b**, The second small homoeo domain in seven homoeobox genes from man (*HHO.c13*, *c1* and *c8*), *Drosophila* (*Antp*, *Dfd* and *cad*) and *Xenopus* (*Xhox-1A*). The pentapeptide domain is boxed. Identical amino acids are in capital letters. **c**, Top: restriction endonuclease map of the *HHO.c13* transcription unit on a genomic clone (λ13G), as determined by Southern blot analysis. Bottom: A, B, C, D indicate contiguous subclones of *HHO.c13* (*Pst*I/*Apa*I, *Apa*I/*Eco*RI, *Eco*RI/*Apa*I, *Apa*I/*Bam*HI), used as probes; black box, homoeobox. A, *Apa*I; Al, *Alu*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H2, *Hind*II; H3, *Hind*III; Hf, *Hinf*II; kb, kilobase pairs.

Methods. Human cDNA clones containing homoeobox sequences from simian virus 40 (SV40)-transformed human fibroblasts²⁴ were subcloned in the pEMBL8 plasmid³⁹ and sequenced as described by Maxam and Gilbert⁴⁰. A genomic DNA clone (λ13G) was isolated from a human genomic library in λ Charon 28, digested with restriction endonucleases, electrophoresed on 0.8–1.5% agarose gels, transferred to nitrocellulose paper by Southern blotting and hybridized to nick-translated *HHO.c13* clone or subclones according to standard techniques⁴¹.



b

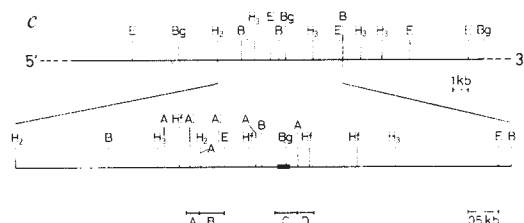
HHO.c13	Leu Lys Gln Pro Ala Val	Val TYR PRO TRP MET	Lys Lys Val His Ala Asn ... 10 AA ...	HOMEO DOMAIN
HHO.c1 (24)	Ala Glu Ser Asn Phe Arg	Ile TYR PRO TRP MET	Arg Ser Ser Gly Thr Asp ...	HOMEO DOMAIN
HHO.c8 (24)	Gln Lys Ala Ser Ile Gln	Ile TYR PRO TRP MET	Gln Arg Met Asn Ser His ... 8 AA ...	HOMEO DOMAIN
Xhox-1A (9)	Pro Glu Gln Asp Pro Val	Val TYR PRO TRP MET	Lys Lys Ala His Ile Ser ... 10 AA ...	HOMEO DOMAIN
Antp (28)	Ser Gly Met Pro Ser Pro	Leu TYR PRO TRP MET	Arg Ser Gln Phe Gly Lys ... 3 AA ...	HOMEO DOMAIN
Dfd (2/26)	Thr Asp Gly Gln Arg Ile	Ile TYR PRO TRP MET	Lys Lys Ile His Val Ala ... 13 AA ...	HOMEO DOMAIN
cad (29)	Ser Pro Ser Lys Pro Pro	Tyr Phe Asp TRP MET	Lys Lys Pro Ala Tyr Pro ... 10 AA ...	HOMEO DOMAIN

sequence is present in the *Drosophila Antp* gene²⁸. The pentapeptides are located upstream from the homoeo domains starting between positions -11 and -24 (Fig. 1b). Similarly, a Trp-Met dipeptide is found at -18 from the unique homoeo domain of *Drosophila caudal* (*cad*) gene²⁹. The pentapeptide apparently defines a second small homoeo domain, located at a short, slightly variable distance from the 5' end of the homoeo domain itself, that is not encoded by the homoeobox containing exon^{9,26,28,29}.

Finally, it is noteworthy that the 23 amino-terminal residues

deduced from *HHO.c13* bear a striking homology to the corresponding peptide sequence encoded by *Xhox-1A*⁹ (Fig. 1a legend).

HHO.c13 was used as hybridization probe to screen a human genomic library in λ Charon 28. A 24-kb genomic clone (λ13G) containing *HHO.c13* was characterized by restriction endonuclease mapping (Fig. 1c) and partially sequenced (not shown). The *HHO.c13* coding region is interrupted by a 541-bp intron, located upstream from the homoeobox (Fig. 1a, c).



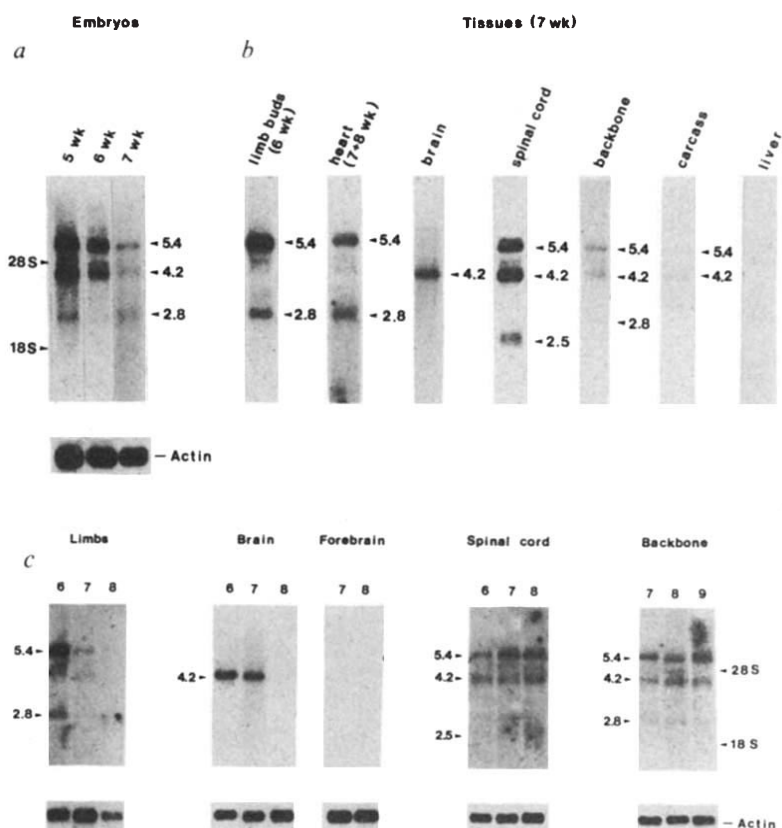


Fig. 2 *a*, Northern blot analysis of the expression of *HHO.c13* in poly(A)⁺ RNA from whole human embryos at 5, 6 and 7 weeks after fertilization. The relative mobility of 28S and 18S rRNA is shown as size markers. Sizes are in kb. *b*, Expression of *HHO.c13* in poly(A)⁺ RNA obtained from pools of organs or body parts dissected from human embryos of 7 weeks. Limb buds were obtained at 6 weeks, hearts pooled at 7 and 8 weeks. Brain was dissected from spinal cord at the level of the pontine flexure, separating metencephalon from myelencephalon. 'Carcass' indicates poly(A)⁺ RNA from a control embryo deprived of limbs, brain and spinal column. *c*, Expression of *HHO.c13* in limb buds or limbs, brain, spinal cord and backbone from human embryos of 6, 7, 8 and 9 weeks, as analysed by Northern blot hybridization of poly(A)⁺ RNA. Sizes are in kb. The forebrain contains the cerebral hemispheres, dissected from midbrain (mesencephalon) and hindbrain (metencephalon).

Methods. Total RNA was extracted by the guanidinium thiocyanate technique⁴², poly(A)⁺-selected by one passage on oligo (dT)-cellulose columns, run in 2–3 µg aliquots on 1.0% agarose-formaldehyde gels⁴¹ and transferred to nitrocellulose or nylon membranes by Northern capillary blotting⁴³. Filters were hybridized to 10⁷ c.p.m. of *HHO.c13* probe labelled by nick-translation to specific activity of 3–6 × 10⁸ d.p.m. per µg, washed at high stringency (final wash 0.1 × SSC, 0.1% SDS at 60 °C) and exposed for 1–7 days to Kodak XR-5 X-ray films in X-omatic intensifying screen cassette. Filters were rehybridized to a chicken β-actin probe⁴⁴ for normalization.

Chromosome mapping by standard procedures indicates that the *HHO.c13* gene is localized on the long arm of chromosome 2 (K. Hübner and C. Croce, personal communication). These data indicate that the *HHO.c13* clone defines a new human homoeobox gene, unrelated to the cluster previously identified on chromosome 17^{12,13}.

We then used the *HHO.c13* cDNA clone as a probe to analyse the expression of the gene in human embryonic and early fetal development. Embryos and fetuses were obtained virtually intact from legal curettage abortions, from 5–9 weeks after fertilization³⁰. The age of embryos (5–8-week-old) was established by morphological staging according to multiple criteria³¹; the youngest were from the first part of the fifth week (total length 7–9 mm, developmental stage 13–14). Fetuses (9-week-old) were staged on the basis of standard age/crown-rump length (CRL) plots³². The dating error was about ±2 days (see also refs 25, 30, 31).

Northern blot analysis of poly(A)⁺ RNA obtained from whole embryos at 5, 6 and 7 weeks (Fig. 2*a*) indicates that the *HHO.c13* gene is expressed in at least three transcripts of 5.4, 4.2 and 2.8 kb: their abundance, however, sharply declines between the fifth and seventh weeks. No signal was detected in Northern blots of corresponding poly(A)[−] RNA (not shown). Poly(A)⁺ RNA was probed with cDNA subclones with or without the homoeobox (probes C or B and D respectively, see Fig. 1*c*): in all cases the pattern was virtually identical (not shown), thus excluding cross-hybridization with transcripts from other homoeobox containing genes. Note that the noncoding strand of the central 170-bp *EcoRI*–*BglII* fragment of *HHO.c13* (see Fig. 1*a*) detects all three major transcripts, whereas the coding strand does not show any major hybridization band (data not shown).

We then analysed the expression of *HHO.c13* in different organs or body parts, dissected by microsurgery from 7-week-old embryos (CRL 16–20 mm, stage 18–19); samples from different specimens were pooled when necessary. Limb buds were obtained at 6 weeks (CRL 10–15 mm, stage 16–17), hearts pooled

at 7–8 weeks (CRL 20–20 mm, stage 20–23). Brain and spinal cord, dissected free of contaminating tissues, were separated at the level of the pontine flexure, which delimits metencephalon from myelencephalon (*medulla oblongata*). As shown in Fig. 2*a*, *b*, human embryos show a differential, tissue-specific pattern of expression of the *HHO.c13* gene, both quantitatively and qualitatively. In particular, (1) 5.4- and 2.8-kb transcripts are predominantly expressed in limb buds and heart; (2) only the 4.2-kb band is observed in brain, but (3) 5.4-, 4.2- and 2.5-kb transcripts are detectable in spinal cord, the last showing the characteristic broad appearance of other cord-specific homoeobox-containing transcripts (ref. 25 and our unpublished results); (4) 5.4-, 4.2- and a faint 2.8-kb bands are observed in the backbone rudiment; (5) 4.2- and to a lesser extent 5.4-kb transcripts are detectable in RNA from control embryos ('carcass'), deprived of limbs, brain and spinal column; (6) no signal is observed in liver.

To define whether the expression of *HHO.c13* transcripts is stage-specific, we have analysed poly(A)⁺ RNA from limb buds or limbs, brain, spinal cord and backbone at 6, 7 and 8 weeks (Fig. 2*c*).

Expression of the 5.4- and 2.8-kb transcripts in limbs declines dramatically between the sixth and the seventh weeks, and almost disappears by eight weeks. This correlates with the morphogenetic progression of limb development, which starts late in the fifth week and is almost complete at the end of the eighth week³¹. Significant expression of *HHO.c13* transcripts is thus confined to the limb bud development stage.

The 4.2-kb transcript observed in the brain is expressed at constant or possibly slightly declining level between six and seven weeks, but almost disappears by eight weeks. Dissection of forebrain (developing cerebral hemispheres) from midbrain (mesencephalon) and hindbrain (metencephalon) indicates that at seven weeks expression of this transcript is confined to the two latter structures. We could not discover whether this spatial restriction also occurs at 6 weeks; if so, it might be related to development of well-defined structures in the 6–7 week period (for example, the cerebellum).

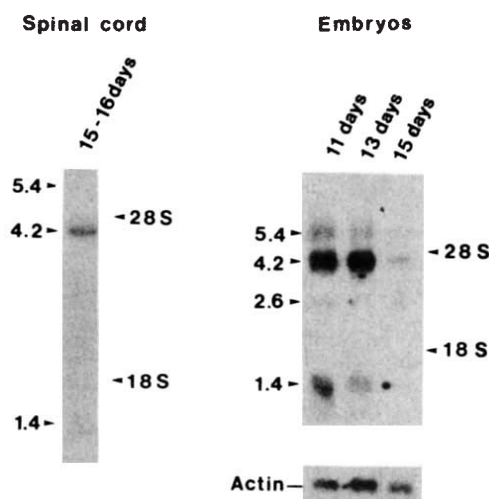


Fig. 3 Northern blot analysis of the expression of a HHO.c13 rat homologue. Poly(A)⁺ RNA was obtained from both spinal cord at 15–16 days from fertilization (5 µg per lane, left panel) and embryos *in toto* at 11, 13 and 15 days (3 µg, right panel). Relevant transcripts are indicated. Sizes are in kb.

Table 1 Homologies between the HHO.c13 homoeobox and the corresponding region of other genes or clones, at nucleotide and amino-acid level

Genes or clones	Nucleotides (%)	Amino acid (%)	Reference
Human			
HHO.c1	72.0	82.0	24
HHO.c8	72.0	77.0	24
HHO.c10 (= <i>Hu1</i>)	74.3	90.1	25, 23
Hu2	68.3	78.7	23
Mouse			
m5	69.9	80.3	18
m6	74.3	82.0	18
Mo10	71.0	75.4	10
Hox 1–3	81.9	98.3	21
Hox 3	66.1	63.9	19
Xenopus			
AC1	68.3	77.0	8
MM3	72.6	83.6	8
Xhox-1A	76.5	93.4	9
Xhox-1B	73.8	90.0	9
Drosophila			
<i>Antp</i>	72.0	83.6	45
<i>ftz</i>	68.3	77.0	45
<i>Ubx</i>	65.6	70.5	45
<i>Dfd</i>	73.2	90.2	27, 26
<i>Scr</i>	72.7	87.9	46, 27, 26
<i>iab-7</i>	58.2	54.1	27
<i>cad</i>	57.9	55.7	29
Sea urchin			
HB1	66.7	77.0	47

Expression of 5.4-, 4.2- and 2.5-kb transcripts in the spinal cord, and 5.4-, 4.2- and 2.8-kb transcripts in the developing backbone, appear constant throughout the 6–8 and 7–9 week period respectively. It is worth noting that expression of at least one other homoeobox gene (HHO.c10 or *Hu1*) is apparently constant in the spinal cord in the same period of human embryogenesis²⁵.

Earlier stages of development were explored in the rat. We first analysed poly(A)⁺ RNA from spinal cord of rat embryos at 15–16 days from fertilization, corresponding to ~7 weeks in human development. As shown in Fig. 3, HHO.c13 hybridizes

to at least three transcripts of 5.4-, 4.2- and 1.4-kb, presumably from a rat gene homologous to HHO.c13. We then extended the analysis to poly(A)⁺ RNA from whole rat embryos at developmental stages from day 11 (total length 1.5–2.0 mm, 13 somites) to 15 (CRL 9 mm, >40 somites)³³, (approximately the beginning of the fourth and the end of the sixth week of human embryogenesis)³¹. Transcripts of 5.4-, 4.2-, 2.6- and 1.4-kb were observed (Fig. 3), with constant expression from day 11–13 decreasing by 50% at day 15. Thus the HHO.c13 gene is already active in rat (and possibly human) embryos at a stage of development immediately after the complete fusion of the neural tube.

The transcripts from human embryos are significantly longer than the cDNA clone (isolated from a simian virus 40 (SV40)-transformed adult fibroblast cDNA library³⁴). Preliminary mapping data on embryonic RNAs (not shown) indicate that the major 5.4- and 4.2-kb transcripts terminate ~2 kb downstream from the HHO.c13 polyadenylation site, whereas 2.8-kb transcripts do not extend further downstream. If our cDNA clone is the 3' portion of the 2.8 kb transcript, it may be suggested that this transcript has an unusually long 5'-UT sequence.

In *Drosophila* the homoeobox genes, mainly organized in multigene clusters, are involved in the control of crucial developmental processes, as the determination of the number and fate of individual body segments¹. In amphibians and mammals homoeobox genes, often organized in multigene clusters^{15,18,21,23}, might act similarly. Studies on early mammalian development show that a variety of homoeobox genes are expressed in murine^{14–18,21,22} and human²⁵ embryos, often with a time-related pattern^{14–18,21,22}. Analysis of single organs from mammalian embryos or newborns reveals selective expression of homoeobox genes in the spinal cord^{14,19,25}, which typically constitutes a metameric structure: in particular, *Hox-3* is expressed in a spatially restricted pattern¹⁹, reminiscent of that observed for other homoeobox genes in *Drosophila*^{35,36}.

Our observations suggest that homoeobox genes may exert a wide spectrum of control functions in the embryonic and early fetal development of mammals, not only in the spinal cord, but also in a variety of other organs and body parts that follow basically different patterns of morphogenesis.

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Cross-regulatory interactions among the gap genes of *Drosophila*

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Segmentation of the *Drosophila* embryo involves expression of several classes of zygotic genes in response to positional information of maternal origin¹. Zygotic expression of the gap genes is thought to be required for the subdivision of the embryo into several units of adjacent segments; pair-rule genes establish a repeat unit length of two segments and segment polarity genes specify parts of the single segment²⁻⁵. The positional information provided by the three classes of segmentation genes is interpreted for the establishment of segment identity using the homoeotic genes⁶⁻¹⁰. Molecular probes for several segmentation and homoeotic genes reveal a temporal and spatial pattern of gene

expression in the young embryo¹¹⁻¹⁸. Changes in the pattern of expression of these genes in mutant embryos provide evidence for a network of interactions among homoeotic and pair-rule genes¹⁹⁻²³ that itself depends on the activity of gap genes²⁴⁻²⁷. We show here that the spatial limit of gap gene expression depends on bilateral interactions among gap genes.

Gap mutant embryos lack a number of adjacent segments which define the domains of the wild-type gene requirement. Based on the deletion pattern (Fig. 1), zygotic *hunchback* (*hb*) activity is required for normal establishment of gnathal and thoracic segments (up to T3), *Krüppel* (*Kr*) for thoracic and anterior abdominal segments (T1-A5), and *knirps* (*kni*) for the abdominal segments A1 to A7 (ref. 2). In a first and naive view, one might expect expression of gap genes in overlapping domains which themselves correspond to the deleted regions in the mutant phenotypes, as shown before for members of the pair-rule class of segmentation genes^{13,14,18}. According to a theoretical model by Meinhardt²⁸, however, the expression of gap genes in response to positional information of maternal origin occurs in adjacent, non-overlapping domains. Each domain should be smaller than the deleted region in the mutant embryo. The limits of the regions where gap genes are expressed (at blastoderm stage) would then provide additional positional cues for the expression of other segmentation genes in a hierarchical order. The model predicts further that these borders

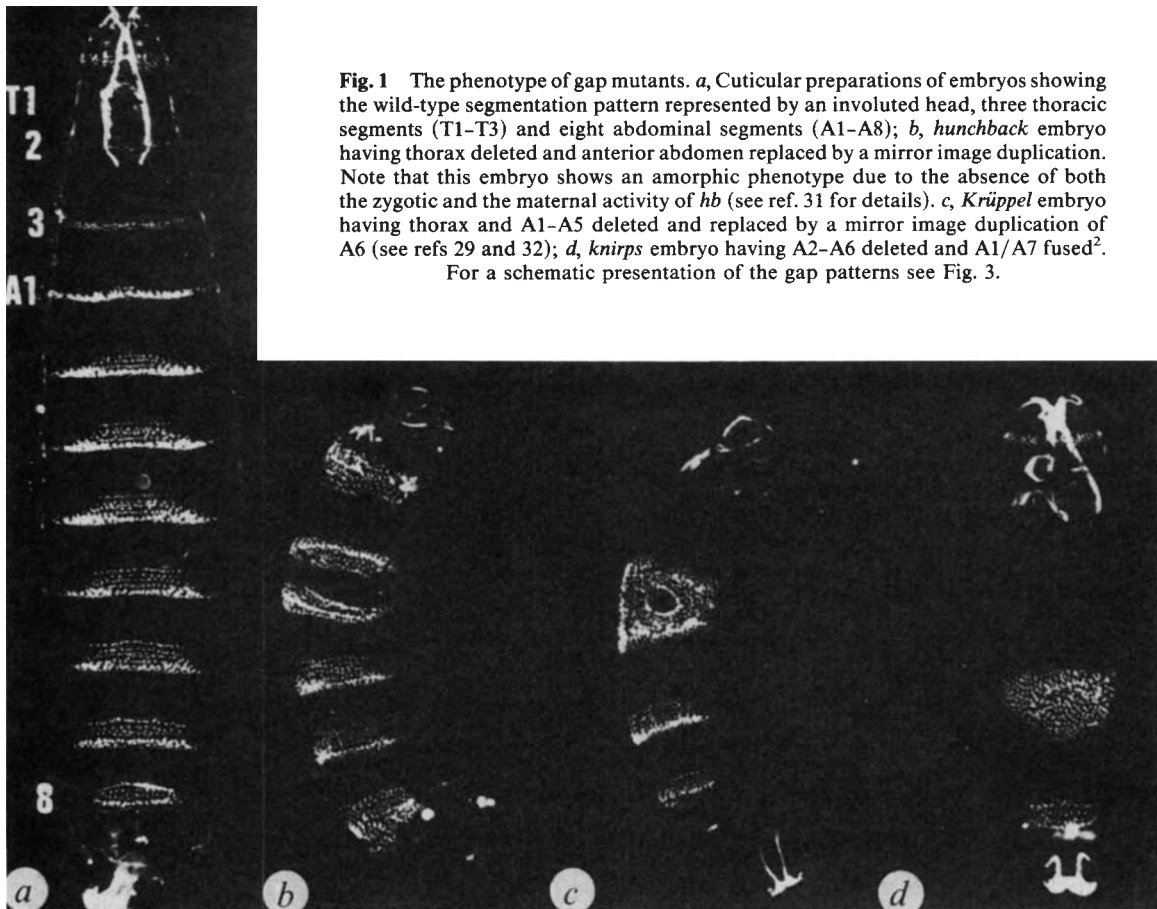
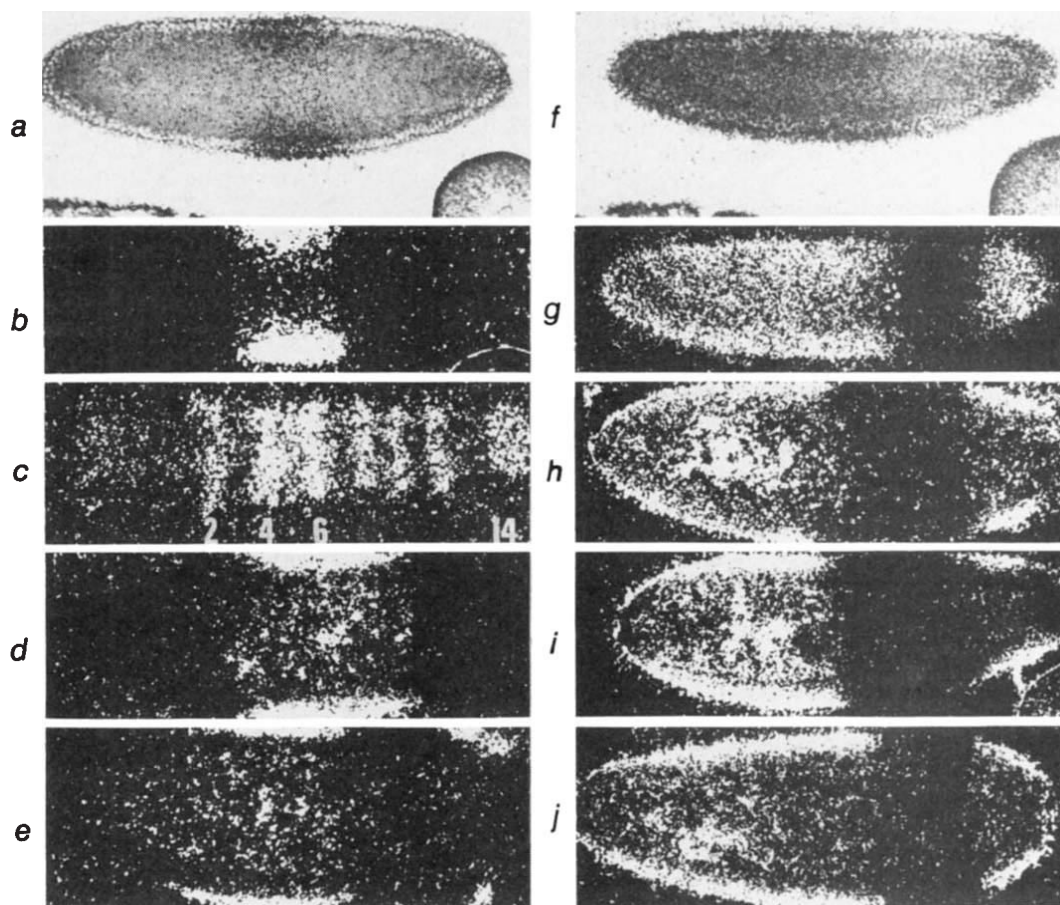


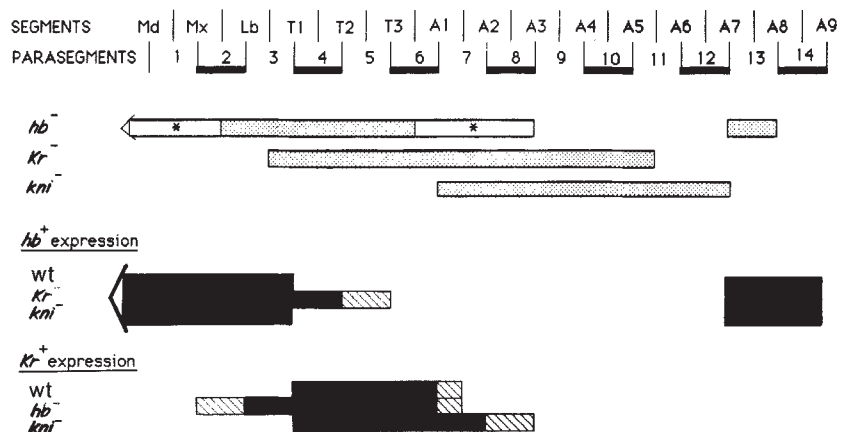
Fig. 1 The phenotype of gap mutants. *a*, Cuticular preparations of embryos showing the wild-type segmentation pattern represented by an involuted head, three thoracic segments (T1-T3) and eight abdominal segments (A1-A8); *b*, *hunchback* embryo having thorax deleted and anterior abdomen replaced by a mirror image duplication. Note that this embryo shows an amorphic phenotype due to the absence of both the zygotic and the maternal activity of *hb* (see ref. 31 for details). *c*, *Krüppel* embryo having thorax and A1-A5 deleted and replaced by a mirror image duplication of A6 (see refs 29 and 32); *d*, *knirps* embryo having A2-A6 deleted and A1/A7 fused². For a schematic presentation of the gap patterns see Fig. 3.

Fig. 2 *In situ* localization of Kr^+ and hb^+ transcripts in gap mutant embryos after the thirteenth nuclear division. *a, b*, Kr^+ expression in blastoderm stage wild-type embryos; *a*, bright field photograph of a parasagittal section and *b*, corresponding dark-field photograph to visualize the *in situ* hybridization pattern of the Kr probe. Note that even after overexposure, the Kr^+ anterior domain remains within the borders of 12–14 cells¹⁷. *c*, *In situ* pattern of a mixed *fushi tarazu* (*ftz*) and Kr^+ probe hybridized to the tangential section of the embryo shown in *a*. Note that the Kr^+ domain (low specific activity) overlaps *ftz*-stripes 2 and 4 and thus corresponds to the approximate limits of parasegments 4–6 (indicated by numbers, ref. 14). Kr^+ expression in *d*, *kni*⁻ and *e*, *hb*⁻ embryos. Note the broadening of Kr^+ expression into the adjacent gap gene domain if the corresponding gene activity is absent. *f*, Bright field and *g*, corresponding dark field photograph of wild type section between *a* and *c* which was hybridized with a mixed hb^+/Kr^+ probe. *h*, parasagittal section (dark field) of the same embryo hybridized with the hb^+ probe. Expression of hb^+ in: *i*, *kni*⁻ and *j*, Kr ⁻ embryos. Note a broadening of the hb^+ domain in Kr ⁻ but not in *kni*⁻ embryos. Orientation of embryos is anterior left and ventral bottom. Note that yolk cells express both Kr^+ and hb^+ (ref. 17, and D.T., unpublished).



Methods. Embryos from wild-type, Kr AP1/SM1 (ref. 29), *kni* FC13/TM3 or *hb* Df(3R)p13/TM3 (ref. 33) parents were collected within 2 h after deposition and kept for 1 h at 26 °C. Serial sections were prepared and subsequently handled as described¹⁷, except that they were hybridized *in situ* using nick-translated ³⁵S-labelled hb^+ and/or Kr^+ probes (2.5×10^7 c.p.m. per μ g DNA). Exposure was 10–20 days in the cold. For positional reference in the embryo, the mixed *ftz*/ Kr^+ probe was hybridized to wild-type and gap mutant sections alternating with Kr^+ and/or hb^+ hybridized sections (see Fig. 3 legend). This allowed, in addition to statistics, hb^- embryos to be recognized by the lack of hb^+ transcripts and a changed *ftz*⁺ pattern²⁶; Kr^- by the lack of Kr^+ transcripts and a changed *ftz*⁺ pattern²⁶. Embryos which were *kni*⁻ were identified by the broadened Kr^+ domain in about a quarter of embryos which have the changed *ftz*⁺ pattern²⁶. The limit of resolution of Kr^+ and hb^+ expression on our sections is about two cells.

Fig. 3 Summary of the spatial distribution of hb^+ and Kr^+ expression in gap mutant embryos. Schematic diagram of the patterns of hb^+ transcripts in wild type (wt), Kr^- and *kni*⁻ embryos, and Kr^+ transcripts in wild type (wt), hb^- and *kni*⁻ embryos, respectively. Black bars, areas of expression, dashed areas, subjective limits of resolution. Above, the deletion of adjacent segments in the different gap mutant embryos is indicated by dotted bars. Asterisks, gap extension in *hb* embryos in the absence of the maternal hb^+ complement³¹. For a positional reference of Kr^+ and hb^+ domains in wild-type embryos, segments and parasegments 1–14 were determined by use of the *fushi tarazu* probe, which is localized in parasegments 2, 4, etc. (thin black bars, see Fig. 2c) as described¹⁴. The analysis is within the limits of two cells and it is based on a minimum of 16 embryos with the respective genotype. Md, mandibular segment; Mx, maxillary segment; Lb, labial segment; T1–T3, thoracic segments; A1–A8, abdominal segments.



are established by interaction between pairs of gap genes, the activities of which are maintained in their domains by autocatalytic and competitive processes²⁸.

Recent cloning of *Kr*²⁹ and *hb* (D.T., unpublished) provides molecular probes to analyse the spatial pattern of *Kr*⁺ and *hb*⁺ expression by *in situ* hybridization to gap mutant embryos (Fig. 2). Adjacent sections of early blastoderm stage embryos were probed with a mixed *Kr*⁺/*hb*⁺ (Fig. 2f,g) or *Kr*⁺/*fushi tarazu* probe to provide positional reference points. This allowed us to map the 12–14 cell wide domain of *Kr*⁺ expression in parasegments 4–6 and the anterior domain of *hb*⁺ expression (Fig. 2j) anterior to *Kr* (Fig. 2a–c; f–h). The size and the borders of the *Kr* and the *hb* domains were changed in gap mutant embryos, but only if the activity of the gap gene in the adjacent domain was missing. In amorphic *kni*[−] embryos, *Kr*⁺ expression extends 6–8 cells posterior to its normal boundary (Fig. 2d), into the region corresponding to parasegment 8 or segment A3 in the wild type embryo (Fig. 3). Expression of *hb*⁺, in contrast to *Kr*, appears unchanged in *kni*[−] embryos (Fig. 2i). But the *hb*⁺ domain broadens 6–8 cells posteriorly if *Kr*⁺ activity is absent (Fig. 2j). It forms a new posterior boundary in a region which is normally parasegment 5 or segment T3 (Fig. 3). *Kr*⁺ expression in *hb*[−] embryos has a complementary effect; the *Kr*⁺ expression extends 6–8 cells anteriorly, if *hb*⁺ activity is missing in the embryo (Fig. 2e). The new anterior boundary of *Kr*⁺ appears in a region which corresponds to parasegment 2 or maxilla in normal embryos (Fig. 3). These results indicate that the normal domain of gap gene expression is dependent on gap gene activity in the adjacent domains. This suggests cross-regulation of gap gene activity at the same hierarchical level as has been proposed as a key prediction in Meinhardt's model²⁸.

There is a second, posterior domain of *Kr*⁺ (at late blastoderm; see details in ref. 17) and *hb*⁺ expression (Fig. 2; and D.T., unpublished) that relates to the fusion of abdominal segments A7 and A8 in late *hb*[−] embryos, and to the lack of malpighian tubules in late *Kr*[−] embryos. The spatial characteristics of these posterior domains remain unaffected in each of the gap mutant embryos analysed (Fig. 2 and data not shown). This suggests that the control of expression in the posterior domain is different from anterior, possibly depending on different maternal cues, other gap genes which may act in that region and/or a feedback control by the activities of other segmentation genes. Such activities may also be required to establish the new anterior and posterior limits of *Kr*⁺ activity in *hb*[−] and *kni*[−] embryos, respectively. Neither of these boundaries have any obvious morphological correspondence in the phenotype of any known gap mutant embryo. Thus the *hb* posterior border in *Kr*[−] embryos may relate to the complementary extension of the *kni*⁺ domain and hence an interaction of *hb* and *kni* in the absence of the *Kr*⁺ gene product, as predicted by Meinhardt²⁸.

We do not understand the molecular basis of these interactions between gap genes. Because the *Kr* gene product shares homology with the DNA-binding domain of transcription factor³⁰, it is tempting to speculate that gap gene products may regulate each other's activity through mutual or competitive DNA-binding interactions as has been suggested for cross-regulation of the expression of homoeobox containing genes^{21,22}.

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Location of a δ -subunit region determining ion transport through the acetylcholine receptor channel

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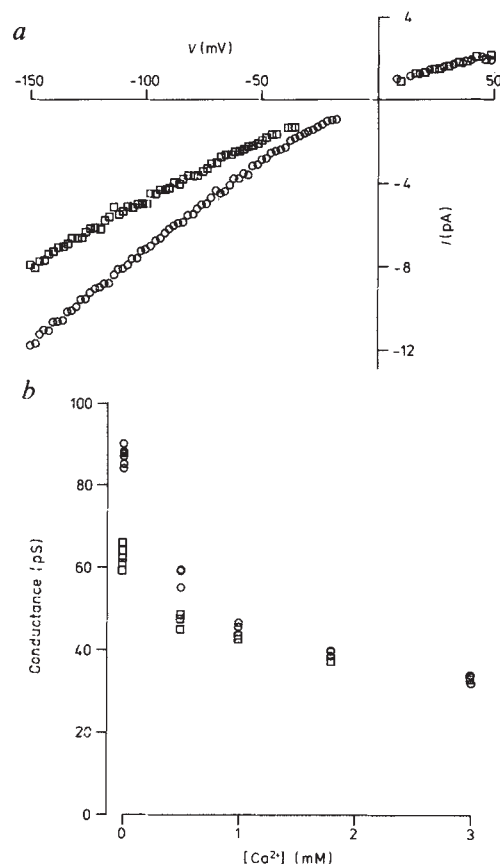
The combination of complementary DNA expression and single-channel current analysis provides a powerful tool for studying the structure-function relationship of the nicotinic acetylcholine receptor (AChR) (refs 1–5). We have previously shown that AChR channels consisting of subunits from different species, expressed in the surface membrane of *Xenopus* oocytes, can be used to relate functional properties to individual subunits⁴. Here we report that, in extracellular solution of low divalent cation concentration, the bovine AChR channel has a smaller conductance than the *Torpedo* AChR channel. Replacement of the δ -subunit of the *Torpedo* AChR by the bovine δ -subunit makes the channel conductance similar to that of the bovine AChR channel. To locate the region in the δ -subunit responsible for this difference, we have constructed chimaeric δ -subunit cDNAs with different combinations of the *Torpedo* and bovine counterparts. The conductances of AChR channels containing chimaeric δ -subunits suggest that a region comprising the putative transmembrane segment M2 and the adjacent bend portion between segments M2 and M3 is involved in determining the rate of ion transport through the open channel.

The *Torpedo* and bovine AChRs were expressed in *Xenopus laevis* oocytes by microinjection of the respective α -, β -, γ - and δ -subunit-specific messenger RNAs synthesized by transcription *in vitro* of the cloned cDNAs (refs 2, 4, 6). Figure 1a compares the sizes of acetylcholine(ACh)-activated elementary inward currents mediated by the *Torpedo* (round symbols) and bovine

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Fig. 1 Conductance properties of the *Torpedo* and bovine AChR channels produced in *Xenopus* oocytes. **a**, Single-channel *I-V* relations of (○) *Torpedo* and (□) bovine AChR channels injected with the respective α -, β -, γ - and δ -subunit-specific mRNAs. All single-channel *I-V* relations were measured at $12 \pm 1^\circ\text{C}$ in cell-attached patches. The pipette was filled with 30 mM KCl, 70 mM KF, 1 mM MgCl_2 , 10 mM EGTA and 10 mM HEPES (pH 7.2); the concentration of free Mg^{2+} is calculated to be less than $1 \mu\text{M}$, using a stability constant for MgEGTA of $1.6 \times 10^5 \text{ M}^{-1}$. This solution is referred to as low divalent cation extracellular solution (LDCS). Each point represents the mean amplitude of at least four elementary currents measured during either rampwise or stepwise changes of the voltage applied to the patch pipette. In experiments with LDCS in the pipette, *I-V* relations were sometimes slightly bent in the inward direction at potentials more negative than -150 mV . Conductances were measured as slope conductances between -40 mV and -150 mV membrane potential by linear regression. The conductances of the *Torpedo* and bovine AChR channels illustrated are 88 pS ($r^2 = 0.997$) and 61 pS ($r^2 = 0.995$), respectively. In experiments with low K^+ pipette solution (10 mM KCl, 5 mM EGTA and 10 mM HEPES (pH 7.2)), the slope conductances were measured at membrane potentials between -90 mV and -200 mV . **b**, Ca^{2+} concentration dependence of single-channel K^+ conductances. Slope conductances measured between -40 mV and -150 mV membrane potential are shown as a function of Ca^{2+} concentration in the pipette, LDCS as in **a**. In experiments with different Ca^{2+} concentrations, CaCl_2 was added to 100 mM KCl and 10 mM HEPES (pH 7.2) to give the desired Ca^{2+} concentration. Each point represents measurement of the slope conductance in one patch. ○, *Torpedo* AChR channels; □, bovine AChR channels.

Methods. The *Torpedo* and bovine AChR subunit-specific mRNAs were synthesized *in vitro* as described previously^{2,4,6}, except that the transcription was primed¹⁹ with the cap dinucleotide $\text{G}(5')\text{ppp}(5')\text{G}$ (0.5 mM) and that the bovine δ -subunit-specific mRNA was synthesized using *EcoRI*-cleaved pSPc82 as template (for construction, see Fig. 3 legend). *Xenopus laevis* oocytes were injected with specified combinations of the *Torpedo* and bovine AChR subunit-specific mRNAs and incubated for 3–5 days⁴. Single-channel current measurements were made from cell-attached membrane patches as described²⁰. The oocyte membrane potential was measured by an intracellular microelectrode and added to the pipette potential to give the patch transmembrane potential. The potential across the patch was voltage clamped via the patch pipette to the desired command potential. In each experiment, the current step sizes were measured during rampwise or stepwise changes of the patch membrane potential between -175 mV and 75 mV . Each *I-V* relation is based on 300–1,400 amplitude determinations in the voltage range between -150 mV and 50 mV . Current records were sampled at 20 kHz after low-pass filtering at 2 or 3 kHz (-3 dB) and analysed by a semiautomatic procedure. The patch membrane potential was sampled at 0.6 kHz . Single-channel current amplitudes within 2 mV intervals were pooled.



(square symbols) AChR channels as measured in cell-attached patches with a potassium-rich pipette solution at low ($<1 \mu\text{M}$) divalent cation concentration. The single-channel current-voltage (*I-V*) relations show that the K^+ conductance of the *Torpedo* AChR channel ($87 \pm 2 \text{ pS}$, mean $\pm$ s.d.), measured as slope conductance between -40 mV and -150 mV membrane potential, is larger than that of the bovine AChR channel ($62 \pm 2 \text{ pS}$) (Fig. 3b). When the Ca^{2+} concentration in the pipette is increased, the conductances of both the *Torpedo* and bovine AChR channels decrease and the two tend to equalize (Fig. 1b). At Ca^{2+} concentrations $\geq 1.8 \text{ mM}$, both channels are equally conductive. A decrease in the conductances of the *Torpedo* and bovine AChR channels was observed in the presence of added extracellular Ca^{2+} with Na^+ , Cs^+ or Li^+ as the permeant cation. The reduction in single-channel conductance was also observed when the extracellular divalent cation was Mg^{2+} , Sr^{2+} or Ba^{2+} . For both channels the effect of Mg^{2+} was more pronounced than that of the other divalent cations. The K^+ conductances at low extracellular divalent cation concentration represent high concentration limits of these conductances since varying the K^+ concentration in the pipette between 10 mM and 100 mM altered the channel conductance of both the *Torpedo* and the bovine AChR by $<10\%$.

One explanation for the dependence of the AChR channel conductance on extracellular divalent cations is that it reflects changes in the effective negative surface charges on the receptor^{7,8}. At low divalent cation concentration, permeant cations would be concentrated at the mouth of the channel as compared with the bulk solution. This concentration effect could cause ion saturation of the channel even when the cation concentration in the bulk solution is low (10 mM). Other mechanisms, such as blocking of monovalent cation flow by divalent cations,

probably play a role at higher divalent cation concentrations⁹. We have used the difference in conductance observed at low divalent cation concentration in an attempt to identify those parts of the AChR molecule important for forming the channel.

To determine whether a particular subunit is responsible for this difference in conductance between the *Torpedo* and bovine AChR channels, we constructed hybrid AChRs by injecting *Xenopus* oocytes with the bovine α -subunit-specific mRNA and

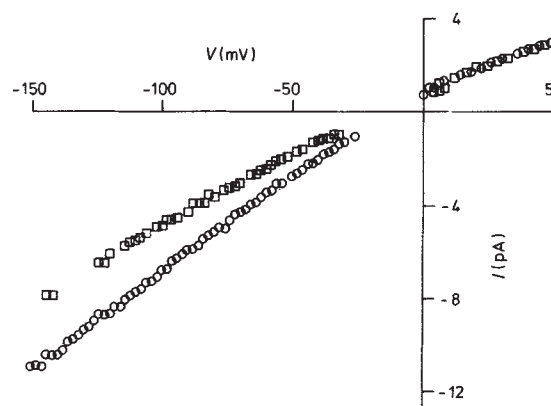


Fig. 2 Single-channel *I-V* relations of α -hybrid (○) and δ -hybrid (□) AChR channels. The hybrid AChR channels were produced in *Xenopus* oocytes by injection of the specified combinations of the *Torpedo* and bovine subunit-specific mRNAs. Experimental details as in Fig. 1a; pipettes filled with LDCS. The slope conductances of the α -hybrid and δ -hybrid AChR channels shown are 84 pS ($r^2 = 0.998$) and 64 pS ($r^2 = 0.996$), respectively.

Fig. 3 Location of a δ -subunit region determining the rate of ion transport through the AChR channel.

a, Structures of chimaeric δ -subunits composed of *Torpedo*²¹ and bovine¹⁵ sequences. The structures of chimaeric δ -subunits, including the amino-terminal signal sequence, are diagrammatically shown such that the hydrophobic segments M1-M4 and the amphipathic segment MA (refs 2, 10-14) are aligned¹⁵; the fact that the bovine δ -subunit is smaller by six amino acids than the *Torpedo* δ -subunit is ignored. Open boxes, amino-acid sequences derived from the *Torpedo* δ -subunit; filled boxes, those from the bovine δ -subunit. Junctional sequences common to the two δ -subunits are indicated by both boxes. The compositions of the individual chimaeric δ -subunits are as follows (T and B denote the *Torpedo* and the bovine δ -subunit, respectively and the numbers in parentheses indicate amino-acid numbers^{15,21}; the junctional sequences common to the two δ -subunits are represented by amino acid numbers of the *Torpedo* δ -subunit): D1, T(-21-350) and B(350-495); D2, B(-21-350) and T(352-501); D16, B(-21-252) and T(254-501); D6, T(-21-231) and B(231-495); D17, B(-21-252), T(254-350) and B(350-495); D8, T(-21-231), B(231-350) and T(352-501); D9, T(-21-231), B(231-284) and T(286-501); D10, T(-21-292), B(292-350) and T(352-501); D11, T(-21-231), B(231-252) and T(254-501); D12, T(-21-256), B(256-284) and T(286-501); D20, B(-21-252), T(254-292) and B(292-495). **b**, Single-channel conductances of the *Torpedo* AChR (T), bovine AChR (B), α -hybrid AChR (α -h), δ -hybrid AChR (δ -h) and AChRs with chimaeric δ -subunits. The AChRs with chimaeric δ -subunits were produced in *Xenopus* oocytes by injection of the respective chimaeric δ -subunit-specific mRNAs together with the *Torpedo* α -, β - and γ -subunit-specific mRNAs. Each point represents measurement of slope conductance in one patch. Experimental details as in Fig. 1a; pipettes filled with LDCS. The mean conductances of the individual AChR channels are as follows: *Torpedo* AChR, 87 ± 2 pS (mean $\pm$ s.d., $n=7$); bovine AChR, 62 ± 2 pS ($n=5$); α -hybrid AChR, 86 ± 2 pS ($n=4$); δ -hybrid AChR, 65 ± 2 pS ($n=6$); AChR-D1, 86 ± 2 pS ($n=5$); AChR-D2, 72 ± 3 pS ($n=6$); AChR-D16, 86 ± 2 pS ($n=6$); AChR-D6, 69 ± 3 pS ($n=6$); AChR-D17, 86 ± 3 pS ($n=5$); AChR-D8, 71 ± 2 pS ($n=6$); AChR-D9, 64 ± 4 pS ($n=5$); AChR-D10, 85 ± 1 pS ($n=6$); AChR-D11, 85 ± 1 pS ($n=5$); AChR-D12, 67 ± 3 pS ($n=5$); AChR-D20, 88 ± 3 pS ($n=8$).

Methods. The 1.113-base-pair (bp) *Sma*I fragment from pSPc δ (ref. 4) was ligated with the synthetic *Hind*III linker 5'-ACAAGCTGTG-3', cleaved with *Hind*III and *Acc*I, and the resulting 423-bp fragment was isolated. The 5.0-kilobase (kb) *Hind*III fragment from pSPc δ was treated with T4 DNA polymerase in the presence of the four deoxyribonucleoside triphosphates, ligated with the synthetic *Eco*RI linker 5'-GGAATTCC-3' and cleaved with *Eco*RI and *Acc*I. The resulting 1.5-kb fragment was ligated with the 423-bp fragment described above and the 3.0-kb *Hind*III-*Eco*RI fragment from pSP64 (ref. 22) to yield pSPc δ 2. The ~830-bp *Sma*I-*Eco*RI fragment from pSPc δ 2, the 4.2-kb *Bam*HI-*Eco*RI fragment from pSP δ (ref. 2) and the synthetic oligodeoxyribonucleotide

5'-GATCTCCATATGTCGCGAGCTGATGAGAGCGAGCAGCGGAT-3'
3'-GAGGTATACAGCGCTCGACTACTCTCGCTCGTCGCGCTA-5'

(prepared with an automatic DNA synthesizer, Applied Biosystems) were ligated to yield pSPD1. The 4.1-kb *Xma*I-*Eco*RI fragment from pSPc δ 2, the ~710-bp *Eco*RI-*Alu*I fragment isolated from the ~770-bp *Eco*RI-*Bam*HI fragment from pSP δ and

5'-CCGGGCGAGACGATTGAG-3'
3'-CGTCTGCTAACTTC-5'

were ligated to yield pSPD2. The 1.1-kb *Hha*I-*Eco*RI fragment from pSP δ , the 3.7-kb *Eco*RI-*Aat*II fragment from pSPc δ 2 and

5'-CACCTTCTACCTCATTATCCGGCG-3'
3'-TGCAGTGAAGATGGAGTAATAGGCC-5'

were ligated to yield pSPD3. The 4.1-kb *Eco*RI-*Sca*I fragment from pSP δ , the ~980-bp *Nco*I-*Eco*RI fragment from pSPc δ 2 and

5'-ACCTGCTCTTTGGCATGGTGGTGGTAC-3'
3'-TGGACGAGAAACCGTACCAACCACTGGTAC-5'

were ligated to yield pSPD4. The 3.4-kb *Acc*I-*Eco*RI and 492-bp *Acc*I-*Nco*I fragments from pSPc δ 2, the ~900-bp *Sca*I-*Eco*RI fragment from pSP δ and

5'-CATGGCCATACCTCTGATTGGTAAGT-3'
3'-CGGTATGCGACTAACCAATCA-5'

were ligated to yield pSPD5. The 678-bp *Sac*I-*Hpa*II fragment from pSP δ and

5'-CGGATAAATTCCTAACGGCACCAATTACAGGAGCT-3'
3'-CTATTTAAGGATTGCGGTGGTTAATGGTCC-5'

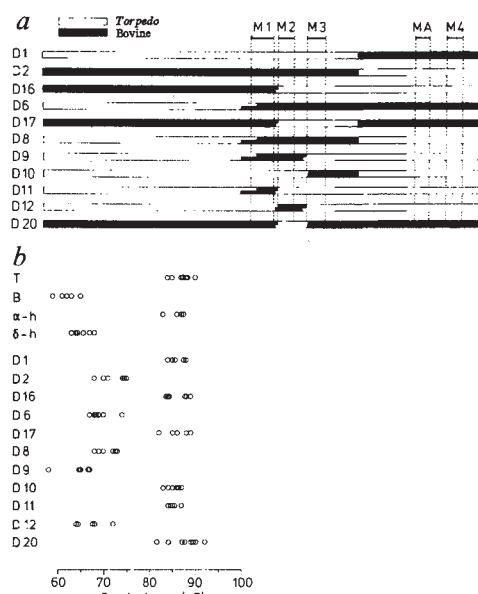
were ligated and cleaved with *Sac*I and *Aat*II. The resulting 715-bp fragment was ligated with the 3.1-kb *Sac*I-*Eco*RI fragment from pSP δ and the 1.2-kb *Aat*II-*Eco*RI fragment from pSPc δ 2 to yield pSPD6. The ~870-bp *Bam*HI-*Eco*RI fragment from pSPD1 and the 4.1-kb *Eco*RI-*Bam*HI fragment from pSPD3 were ligated to yield pSPD7. The ~730-bp *Xma*I-*Eco*RI fragment from pSPD2 and the 4.2-kb *Xma*I-*Eco*RI fragment from pSPD6 were ligated to yield pSPD8. The 3.8-kb *Eco*RI-*Aat*II fragment from pSPD6 and the 1.1-kb *Aat*II-*Eco*RI fragment from pSPD5 were ligated to yield pSPD9. The ~730-bp *Xma*I-*Eco*RI fragment from pSPD2 and the 4.2-kb *Eco*RI-*Xma*I fragment from pSPD4 were ligated to yield pSPD10. The 253-bp *Dde*I-*Bgl*II fragment isolated from the 850-bp *Dde*I fragment from pSP δ was ligated with the 2.1-kb *Bgl*II fragment from pSP δ . The resulting 2.3-kb fragment was ligated with the 2.4-kb *Bgl*II-*Hind*III fragment from pSP δ , the 180-bp *Hind*III-*Sau*3AI fragment isolated from the 228-bp *Hind*III-*Nco*I fragment from pSPD6 and

5'-GATCAACCTGGTCTTCTACTTGGCGGCGAGCTGGTGAGAAAAT-3'
3'-TTGGACCAAGATGAACGGCGGCTGACACCACTCTTTTA-
GAGCAGCGCTATTCTGTCTTGTCTTGC-3'
CTCGTGGCGATAAAGACAGAACGAGGT-5'

to yield pSPD¹. The 4.7-kb *Pst*I-*Bgl*II fragment from pSP δ , the 210-bp *Bgl*II-*Nco*I fragment from pSPD9 and

5'-GAGAGTGGTGACAGACATC-3'
3'-ACGTCTCTACCACTCTTCTGTAGGTAC-5'

were ligated to yield pSPD12. The 326-bp *Acc*I-*Fok*I fragment from pSPD5 and the 328-bp *Fok*I-*Bam*HI fragment from pSPD11 were ligated and cleaved with *Acc*I and *Bam*HI. The resulting 654-bp fragment was ligated with the 4.0-kb *Bam*HI-*Pst*I and 150-bp *Pst*I-*Acc*I fragments from pSPD5 to yield pSPD16. The 326-bp *Acc*I-*Fok*I fragment from pSPD5 and the 328-bp *Fok*I-*Bam*HI fragment from pSPD11 were ligated and cleaved with *Acc*I and *Bam*HI. The resulting 654-bp *Acc*I-*Bam*HI fragment was ligated with the 3.9-kb *Bam*HI-*Hind*III and 423-bp *Hind*III-*Acc*I fragments from pSPD7 to yield pSPD17. The 4.7-kb *Nco*I-*Aat*II fragment from pSPc δ 2, the 153-bp *Aat*II-*Dde*I fragment isolated from the 363-bp *Aat*II-*Bam*HI fragment from pSPD11 and the 102-bp *Dde*I-*Nco*I fragment isolated from the 146-bp *Pst*I-*Nco*I fragment from pSPD4 were ligated to yield pSPD20. Those regions of the constructed plasmids derived from the synthetic oligodeoxyribonucleotides were sequenced²³ to ensure the constructions. The chimaeric δ -subunit-specific mRNAs were synthesized as described in the legend to Fig. 1, using *Eco*RI-cleaved plasmids carrying the respective cDNAs as templates.



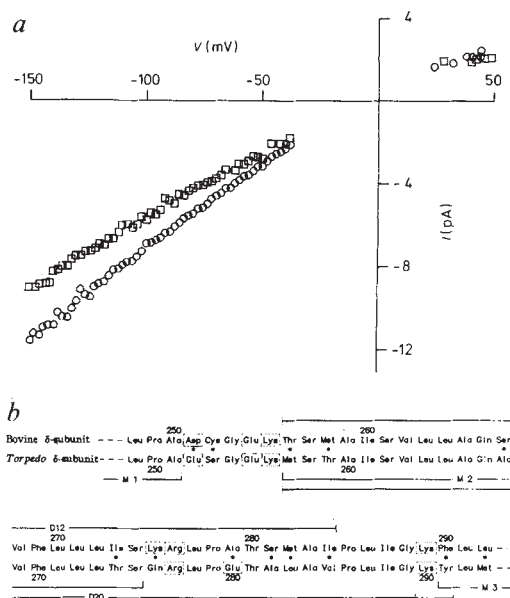


Fig. 4 *a*, Single-channel *I*-*V* relations of the (□) AChR-D12 and (○) AChR-D20 channels. Experimental details as in Fig. 1*a*; pipettes filled with LDCE. The slope conductances of the AChR-D12 and AChR-D20 channels shown are 65 pS ($r^2 = 0.994$) and 85 pS ($r^2 = 0.997$), respectively. *b*, Comparison of the amino-acid sequences of the bovine (top) and *Torpedo* (bottom) AChR δ -subunits in the M2 and adjacent regions. The bovine and *Torpedo* sequences are aligned¹⁵ and the bovine sequence in the D12 subunit and the *Torpedo* sequence in the D20 subunit are indicated. Asterisks, amino-acid differences between the two sequences; negatively charged and positively charged residues are enclosed with solid and dashed lines, respectively. Amino-acid numbers^{15,21} are given and the positions of segments M1, M2 and M3 are indicated; note that the amino-acid numbers of the two δ -subunits do not coincide with the alignment because of a gap present upstream.

the *Torpedo* β -, γ - and δ -subunit-specific mRNAs (α -hybrid AChR, ref. 4) or with the bovine δ -subunit-specific mRNA and the *Torpedo* α -, β - and γ -subunit-specific mRNAs (δ -hybrid AChR, ref. 4). Figure 2 shows the single-channel *I*-*V* relations of the α -hybrid (round symbols) and δ -hybrid (square symbols) AChR channels in low divalent cation extracellular solution. The K^+ conductance of the α -hybrid AChR channel is 86 ± 2 pS (Fig. 3*b*). This is similar to the conductance of the parental *Torpedo* AChR channel, suggesting that a difference in the α -subunit is not responsible for the larger conductance of the *Torpedo* AChR channel. In contrast, the K^+ conductance of the δ -hybrid AChR channel is 65 ± 2 pS (Fig. 3*b*), being similar to that of the bovine AChR channel. Thus, the difference in K^+ conductance between the *Torpedo* and bovine AChR channels can be ascribed, at least partly, to a difference in their δ -subunits. The contributions of the β - and γ -subunits were not assessed because of the very low channel activities of the β -hybrid and γ -hybrid AChRs expressed in *Xenopus* oocytes⁴.

To localize that part of the δ -subunit molecule responsible for the difference in the *Torpedo* and δ -hybrid AChR channel conductances, we prepared cDNA constructs encoding chimaeric δ -subunits in which portions of the *Torpedo* δ -subunit were systematically replaced by homologous regions of the bovine δ -subunit (Fig. 3*a*). The mRNA derived from each of these cDNA templates, together with the *Torpedo* α -, β - and γ -subunit-specific mRNAs, was injected into *Xenopus* oocytes to produce AChRs with the different chimaeric δ -subunits. Figure 3*b* shows graphically the K^+ conductances, measured in low divalent cation solution, of these AChR channels with the different chimaeric δ -subunits. The conductances observed fall into one of two broad classes of lower, bovine-like (64–72 pS) or higher, *Torpedo*-like (85–88 pS) mean conductance.

The conductance of the AChR-D1 channel (86 ± 2 pS) and that of the AChR-D2 channel (72 ± 3 pS) suggest that the portion of the δ -subunit comprising the amino-terminal extracellular region and the putative transmembrane segments M1, M2 and M3 (refs 10–14) is responsible for the different K^+ conductances of the *Torpedo* and δ -hybrid AChR channels. Comparison of the conductances of the AChR-D16 channel (86 ± 2 pS) and the AChR-D6 channel (69 ± 3 pS) further suggests that the critical region lies in the portion containing segments M2 and M3. To refine this mapping, we tested AChRs with δ -subunit chimaeras in which only small segments had been exchanged.

The conductances of the AChR-D17 channel (86 ± 3 pS) and the AChR-D8 channel (71 ± 2 pS) are in agreement with the above localization. Furthermore, the conductances of the AChR-D9, AChR-D10 and AChR-D11 channels (64 ± 4 pS, 85 ± 1 pS and 85 ± 1 pS, respectively) exclude a major involvement of segment M3 as well as of segment M1. Finally, the conductance of the AChR-D12 channel (67 ± 3 pS), in which only the segment composed of amino-acid residues 256–284 of the bovine δ -subunit¹⁵ has been substituted for the *Torpedo* sequence, is similar to that of the δ -hybrid AChR channel (see Fig. 4*a*, square symbols and Fig. 4*b*). In accord with this result is the conductance of the AChR-D20 channel (88 ± 3 pS), which is similar to that of the *Torpedo* AChR channel (see Fig. 4*a*, round symbols). The D20 subunit is nearly a 'mirror image' of the D12 subunit in that only the segment composed of amino-acid residues 256–290 of the bovine δ -subunit has been replaced by the *Torpedo* sequence (Fig. 4*b*). The AChR-D12 and the AChR-D20 channel showed a Ca^{2+} dependence of their conductance similar to that of the bovine and the *Torpedo* AChR channel, respectively.

From these results, we conclude that the region of the δ -subunit comprising segment M2 and the adjacent bend portion between segments M2 and M3 is principally responsible for the difference in K^+ conductance between the *Torpedo* and bovine AChR channels. In line with this conclusion is the finding that replacement of the *Torpedo* α -subunit by the bovine α -subunit, both of which contain an identical amino-acid sequence in this region, does not alter the channel conductance. Thus, our results suggest that the M2 and bend region represents an important determinant of the rate of ion transport through the AChR channel. Recently it has been reported that segment M2 contributes to the binding site of noncompetitive antagonists^{16–18}.

In the bend portion between the putative transmembrane segments M2 and M3, the *Torpedo* δ -subunit has one negative and one positive charge, whereas the bovine δ -subunit has two positive charges; the lysine residue immediately preceding segment M3 is not taken into account because the five amino-acid residues preceding segment M3 are common to the two δ -subunits (Fig. 4*b*). All subunits of both the *Torpedo* and bovine AChRs contain negatively charged residues in this bend portion^{10,15}, except the bovine δ -subunit. A hypothesis consistent with our results is that the negatively charged residues in the bend portion of the AChR subunits are located near the mouth of the channel, attracting permeant cations toward the channel. One effect of substituting the D12 chimaeric subunit for the *Torpedo* δ -subunit would be to reduce the number of negative charges in the mouth region. Alternatively, the M2 and bend region may constitute part of the channel wall and its amino-acid sequence may partly determine the energy profile experienced by permeating cations in the channel.

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Abnormal tyrosine phosphorylation on T-cell receptor in lymphoproliferative disorders

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The study of human autoimmune diseases has benefited greatly from analysis of animal models. Mice that are homozygous for either the *lpr* (lymphoproliferation)¹ or *gld* (generalized lymphoproliferative disease)² mutant genes develop a disease characterized by massive lymphadenopathy and autoantibody formation. With age, the lymphoid organs in these mice are replaced with a greatly expanded population of abnormal lymphocytes³⁻⁵. Recent work has shown that these cells are likely to be in the T-cell lineage. They rearrange and transcribe the genes for the α and β subunits of the T-cell receptor (TCR) and a third, T-cell receptor-like gene, T_γ ^{6,7}. As determined by immunofluorescence with anti-receptor antibodies the cells also express TCR on the cell surface. The murine T-cell receptor consists of the α and β chains, derived from the rearranged α and β genes, in non-covalent association with seven other chains; the δ chain, of relative molecular mass (M_r) 26,000 (26K), the ϵ chain (25K), a glycosylated 21K chain (gp21) which is probably the homologue of the γ chain of T3 (CD3), a 16K homodimer (ζ) and a 21K dimer (p21)⁸⁻¹⁰. This multichain complex is thought to be the murine analogue of the human T3 complex. After activation of normal T cells by antigen or lectin, p21 is phosphorylated on tyrosine residues and gp21 is phosphorylated on serine residues. In contrast, in the *gld* and *lpr* cells, p21 is phosphorylated even in the absence of antigen or lectin, whereas gp21 is not phosphorylated.

The autosomal recessive mutations *lpr* and *gld* are non-allelic genes, yet the abnormal cells that populate the lymph nodes of mice homozygous for either mutation are indistinguishable by a wide variety of criteria. We have suggested that the mutations may affect different components of a common metabolic pathway of major importance to T-cell differentiation and function⁴. The abnormal cells of *lpr* and *gld* mice appear to express a normal complement of clonally determined α - β heterodimers

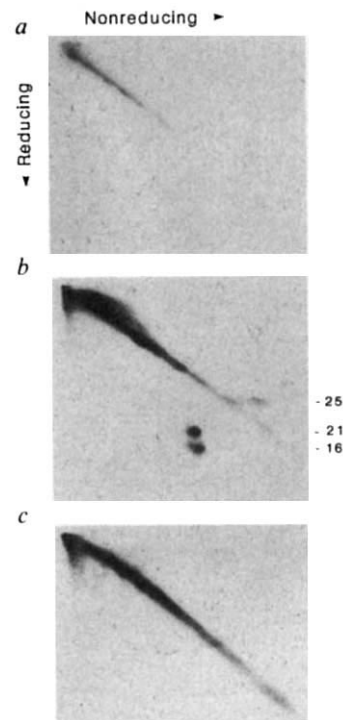


Fig. 1 Two-dimensional diagonal SDS-polyacrylamide gel electrophoresis (PAGE) of phosphorylated normal and *lpr* T cell antigen receptors. Purified Ly2⁺, L3T4⁺ lymph node cells lacking surface immunoglobulin from C3H-*lpr* animals (*a*, *b*) and purified T cells from normal C3H spleens and lymph nodes (*c*) were ³²P-labelled and solubilized in 0.5% Triton X-100. Immunoprecipitations were performed with preimmune rabbit serum (*a*) or with rabbit anti- δ chain serum⁹ (*b*, *c*).

Methods. The abnormal C3H-*lpr* and *gld* T cells (L3T4⁺, Ly2⁺) were separated from normal L3T4⁺ and Ly-2⁺ T cells by panning as described⁷. C3H +/+ T cells were isolated from a mixture of spleen and lymph node cells by incubating 5×10^7 cells on anti-immunoglobulin coated plates for 45 min at 20°C as described previously⁷. Surface-immunoglobulin-negative cells were recovered by gently washing the plates five times. After concentration by centrifugation the cells were applied to a fresh set of anti-Ig coated plates for a second cycle of purification. The cells were washed in phosphate-free RPMI1640, resuspended in this medium with 10% dialysed fetal calf serum at 2.4×10^7 cells per ml and incubated with 0.8 mCi ml⁻¹ [³²P]orthophosphate for 1 h at 37°C. The cells were then washed and solubilized as previously described¹⁰. Immunoprecipitations were performed with 20 μ l of preimmune serum or anti- δ serum adsorbed to protein A-Sepharose. The immunoprecipitates were washed and eluted as described¹⁰. The eluents were electrophoresed in the first dimension (left to right) without reduction in a 13% SDS-PAGE tube gel. The tubes were equilibrated in 0.5% dithiothreitol, 0.1% SDS, 0.125 M Tris pH 6.8 for 2 h at room temperature before electrophoresis in a 13% SDS-PAGE slab gel in the second dimension. Gels were treated and autoradiographed as described¹⁰. Each panel represents immunoprecipitation of lysates from 6×10^7 cells. Molecular weight markers are shown. The gel was exposed to film for 48 h at -70°C.

at the cell surface but fail to respond to stimulation with alloantigens⁷, raising the possibility that the T-cell receptor is involved in the mutation. Therefore, the antigen receptors on the abnormal T cells of *lpr* and *gld* mice were isolated and compared to those of normal peripheral T cells. The cells were purified by plate separation techniques, surface radio-iodinated and solubilized in non-ionic detergents. The antigen receptors were isolated with an antibody that binds the murine antigen receptor δ chain and is capable of immunoprecipitating the entire complex⁹. All the components of the receptor are specifically co-immunoprecipitated with the anti- δ chain reagent from both

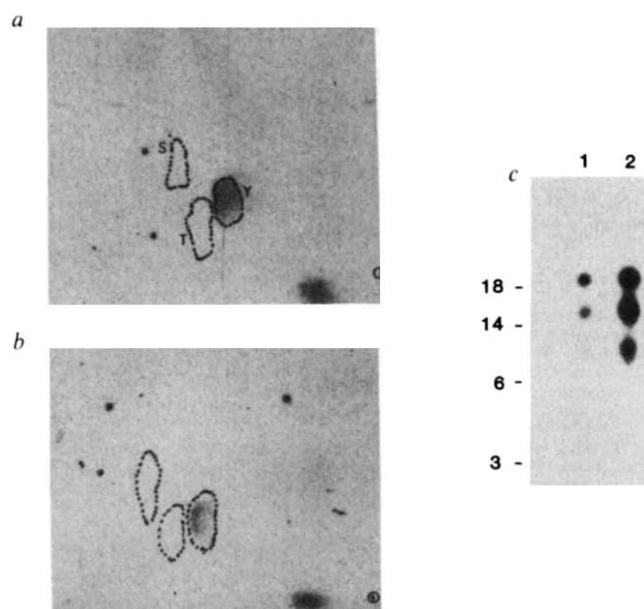


Fig. 2 *a, b*, Phosphoamino acid analysis of phosphorylated antigen receptor subunits from abnormal *gld* cells. Phosphorylated 21K chains (*a*) or 16K chains (*b*) from C3H-*gld* cells were analysed. (*c*) Phosphopeptide mapping of antigen receptor subunits by limited proteolysis. Phosphorylated p21 subunits from antigen activated 2B4 cells (lane 1) and MRL/*lpr* cells (lane 2) were subjected to limited proteolysis with staphylococcal V8 protease.

Methods. Phosphoamino acid content of proteins was determined using a modification of the method of Cooper *et al.*¹⁷. The abnormal cells from C3H-*gld* and C3H-*lpr* lymph nodes were isolated, labeled with ³²P and solubilized as described in Fig. 1. Labeled proteins were separated on two dimensional diagonal gels and detected by autoradiography as described in Fig. 1. Gel regions containing individual proteins were excised and hydrolysed with 2 ml of 5.7 M HCl at 110 °C for 1 h. After addition of 4 ml of water, the hydrolysates were centrifuged at 500g for 10 min, and the supernatants were lyophilized. The residues were dissolved in 10 ml of water, to which was added Dowex AG1-X8 (400 µl of 50% v/v), and phosphoamino acid standards (1 µl of 1 mg ml⁻¹ stock solutions). The pH of the solutions was adjusted to 7.5 to 8.5 with 10 mM NH₄OH. Solutions were tumbled with the ion exchange resin at room temperature for 4 h and centrifuged. The supernatant was reappplied to a second batch of resin and tumbled overnight. The two sets of beads were washed with water and eluted with four sequential 0.5 ml applications of 0.1 M HCl. Eluents were dried under vacuum, pooled in 1 ml of water, redried under vacuum, and stored at 4 °C. Samples were mixed with phosphoamino acid standards and analysed by two dimensional high voltage electrophoresis on thin layer plates as described¹⁶. Electrophoresis was performed at pH 1.9, at 900 V for 1.5 h in the first (vertical) dimension and at pH 3.5, at 900 V for 40 min in the second (horizontal) dimension. Gels were exposed to film for 12 days. The pigeon cytochrome *c* hybridoma 2B4 (2.5 × 10⁸) was ³²P labelled and activated as described elsewhere¹¹. The antigen receptor was isolated with the clonotypic monoclonal, A2B4-2¹⁸. MRL/*lpr* cells (10⁹) were phosphorylated and the antigen receptor was isolated as described in Fig. 1. Phosphorylated components were isolated on two-dimensional diagonal gels, spots containing the p21 chains were cut out, and the polypeptides were subjected to proteolysis with V8 protease in the gel as described¹⁹. A 15% acrylamide gel was used to resolve the phosphopeptides. *a*, 48 h exposure; *b*, 16 h exposure.

normal and abnormal T cell populations (data not shown). Several subunits of the T-cell antigen receptor are phosphorylated during T-cell activation^{8,10-12}. The 21K γ glycoprotein and, to a lesser degree, the 25K ϵ chain are phosphorylated on serine residues, and the p21 structure is phosphorylated on tyrosine residues within minutes of either antigen or mitogen presentation to T cell hybridomas. Several other receptors are also phosphory-

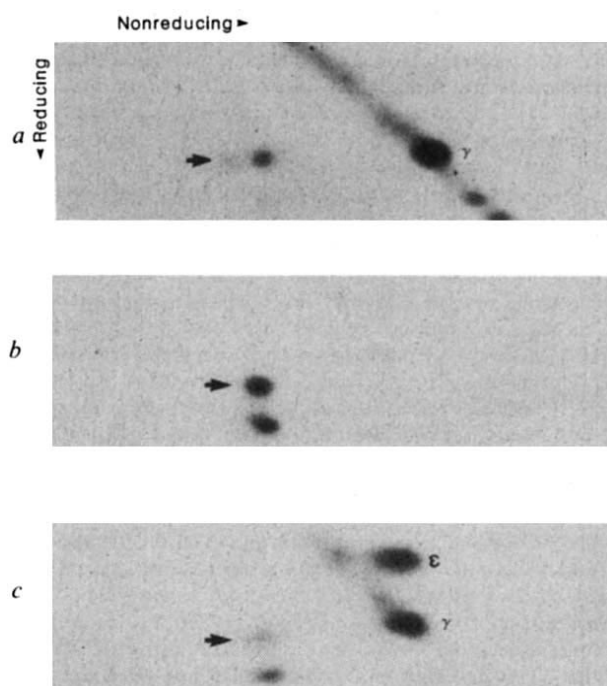


Fig. 3 Two dimensional SDS-PAGE analysis of phosphorylated T cells. Phosphorylated antigen receptors from *a*, antigen-activated 2B4 cells; *b*, C3H-*gld* cells; *c*, PMA treated C3H-*gld* cells were electrophoresed on 2D diagonal gels. Arrow, phosphorylated p21 subunit.

Methods. The pigeon cytochrome *c* specific hybridoma 2B4 was ³²P-labelled and activated by antigen and the antigen receptor was isolated with the clonotypic monoclonal, A2B4-2 as described in Fig. 2*a*. C3H-*gld* cells were isolated as described in Fig. 1*b, c*. After phosphate labelling half of the *gld* cells were treated for 30 min with TPA at 100 ng ml⁻¹ (*c*). Receptors from *gld* cells were isolated with the anti- δ chain antibody. Electrophoresis and autoradiography were as described (Fig. 1).

lated on tyrosine upon activation; tyrosine phosphorylation is otherwise very rare¹³. The serine phosphorylation is presumably due to the activation of protein kinase C and can be mimicked by the addition of TPA (tetradecanoyl phorbol 13-acetate). The kinase responsible for tyrosine phosphorylation has not been identified, but p21 phosphorylation is associated only with presentation of specific antigen or mitogenic lectin. There is little, if any, phosphorylation of receptor chains without activation in normal cells. Surprisingly, in contrast to normal T cells, the *lpr* (Fig. 1) and *gld* T cell (Fig. 3*b*) receptors were heavily phosphorylated in the absence of any added stimulus. Labeled phosphates are added in the course of a one hour incubation. This rapid turnover implies constitutive activation of both a kinase and a phosphatase. Receptors isolated from T cells of animals with either mutation are constitutively phosphorylated on both 21 and 16K chains. The 16K chain may be a proteolytic cleavage of the 21K chain but their exact relationship is still unclear. As these bands are vertically aligned below the diagonal on this two dimensional gel, it is likely that they are linked under non-reducing conditions; they probably form a dimer in the receptor. We have shown that specific antigen activation of T cell hybridomas results in phosphorylation of the p21 component of the antigen receptor on tyrosine residues^{8,12}, so we isolated the 21K and 16K proteins from the *gld* and *lpr* cells to determine which amino acid was phosphorylated. Both chains from the two mutant strains were phosphorylated on tyrosine (Fig. 2*a, b*). Partial phosphopeptide mappings of p21 from *lpr* cells and an antigen-stimulated T-cell hybridoma with V8 protease (Fig. 2*c*) and cyanogen bromide (not shown) are consistent with an identical site of phosphorylation. The differences

between the tyrosine phosphorylations on abnormal and normal T cells cannot be explained by enhanced phosphate exchange. The turnover rate of the p21 tyrosine phosphate in *gld* cells is the same (~20 min) as that seen in activated normal T cells (data not shown). In normal T cells there is no tyrosine phosphorylation on p21 in the absence of stimulation. The constitutive phosphorylation in the *gld* and *lpr* cells must be due to abnormalities in the tyrosine kinase pathway.

In our previous studies, we found that whenever antigen or mitogen activates T cells, two parallel phosphorylation pathways are activated resulting in the serine phosphorylation of gp21 and the tyrosine phosphorylation of p21. A relatively fixed relationship between the intensities of these two phosphorylations is found over a wide range of antigen doses. An example (Fig. 3a) shows that the intensity of the serine phosphorylation is greater than that of tyrosine phosphorylated p21 in a T cell hybridoma. Normal splenic T cells stimulated for 1 hour or 40 hours with the mitogen concanavalin A also show both serine and tyrosine phosphorylations (data not shown). The relative absence of the 16K phosphoprotein in the 2B4 cells appears to be peculiar to this hybridoma as mitogen-stimulated splenic T cells also show the 21K and 16K tyrosine-phosphorylated doublet. In contrast, the *gld* (Fig. 3b) and *lpr* (Fig. 1b) cells demonstrate no detectable gp21 serine phosphorylation despite high levels of p21 tyrosine phosphorylation. The addition of mitogens to *lpr* and *gld* cells failed to enhance the tyrosine phosphorylation and did not result in gp21 serine phosphorylation, again in contrast to normal T cells. This is consistent with the failure to proliferate in response to stimulation with concanavalin A. We examined whether the absence of gp21 serine phosphorylation was due to lack of activatable protein kinase C. Addition of TPA to normal T cells or T-cell hybridomas bypasses the receptor and directly activates this kinase resulting in serine, but not tyrosine phosphorylation of antigen receptor subunits. We therefore added TPA to ³²P-labelled *gld* cells (Fig. 3c). This results in the expected gp21 phosphorylation and enhanced ϵ phosphorylation indicating that protein kinase C is both present and capable of phosphorylating the *gld* gp21 subunit. Interestingly, TPA treatment seems to inhibit phosphorylation of p21.

The constitutive tyrosine phosphorylation in the absence of serine phosphorylation distinguishes the abnormal cells of *gld* and *lpr* from bulk populations of resting splenic T cells, mitogen activated T-cell blasts or antigen-activated T-cell hybridomas, but the fact that the abnormal T cells in these disorders are phenotypically different from the T cells to which they were compared makes the interpretation of these differences difficult. We do not know whether the T cells in these animals are aberrant lineages unique to the disease, or an abnormal expansion of a rare T cells subset present in normal animals. If these cells do exist in normal mice, they might show the same phosphorylation phenomena reported here. The recent report of 'double negative' T cells in the periphery of normal individuals may be due to just such populations^{13,14}. The fact that the T cell antigen receptor plays such a crucial role in the growth and differentiation of T cells makes understanding its function in abnormal T cells essential. The observation that a biochemical pathway identified with receptor activation is constitutively activated in these abnormal T cells may offer clues to the pathogenesis of the disease processes. A defect in the coupling mechanism between the receptor and kinase, or abnormalities in intracellular kinase regulation may be the manifestation of the mutant genes. The clear role of tyrosine kinases in both normal and abnormal growth and differentiation suggests parallels to this system. Current views about the *erbB* oncogene, for example, postulate that its transforming activity is due to the functioning of a constitutively active tyrosine kinase no longer under the regulation of a growth factor hormone^{15,16}. Two observations reported here suggest a similar dissociation from normal ligand regulation: (1) the constitutive tyrosine phosphorylation in the absence of any apparent ligand; and (2) the lack of receptor serine

phosphorylation that characterizes normal ligand induced receptor-activation. Pursuing the implications and consequences of these unusual phosphorylations may elucidate mechanisms involved in both normal and abnormal T cell function.

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Polymorphism of human Ia antigens generated by reciprocal intergenic exchange between two DR β loci

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Class II molecules encoded by the human major histocompatibility complex (MHC) are involved in regulating T-cell response to antigens¹⁻³. The mechanisms for generating polymorphism in products of the MHC have been studied extensively for both the murine H-2 and the human HLA complex. Such studies indicate that point mutations plus selection have a major role in the generation of polymorphisms of class I and class II MHC genes⁴⁻⁸. However, a non-reciprocal gene conversion mechanism has been proposed to explain several examples of clustered sequence variation in MHC genes⁹⁻¹⁴. In all these examples, the proposed gene conversion event is unidirectional; that is, one of the two interacting genes acts as sequence donor and the other as sequence recipient. No examples of potential reciprocal genetic exchange (as occurs in the fungal system^{15,16}), in which the two interacting genes act as both donor and recipient of gene fragments, have been found in the MHC system or in other multigene families of higher organisms. We sequenced two different HLA-DR β complementary DNAs from each of two different cells all expressing the same serologically defined determinant (DR2) but different T-cell-recognized (Dw) specificities (Dw12 and MN2). Sequence comparisons of these four cDNA clones (and two DR β amino-acid sequences from the DR2-Dw2 subtype) suggest that new coding sequences for DR β molecules in the DR2 haplotypes are potentially generated by reciprocal intergenic exchange.

Table 1 lists six amino-acid sequences for DR β genes from three different DR2 subtypes (DR2-Dw2, DR2-Dw12 and DR2-MN2)¹⁷. (For simplicity only amino acids that differ between any two sequences are given; see ref. 18 for a complete listing

Table 1 Grouping and comparison of amino-acid sequences of six DR β -chains from HLA-DR2 individuals

		Amino-acid position																												CP TM CY				
		First domain																Second domain																
Group	β -chain	Dw	6	9	11	13	28	30	31	37	38	47	67	70	71	85	86	96	104	105	108	110	120	133	135	142	150	157	191	203	222	231		
I	Dw2-A	Dw2	R	Q	D	Y	H	D	I	D	L	Y	F	D	R	V	G	E	A	R	T	E	N	R	S	V	N	T	Q	V	K	H		
	DHO-7	Dw12	—	—	—	—	—	—	—	—	—	Y	F	D	R	V	G	E	A	R	T	Q	N	R	S	V	N	—	—	—	—	—		
	MN2-2	MN2	C	Q	D	Y	H	G	I	N	V	Y	I	Q	A	A	V	E	A	R	T	Q	N	R	G	V	N	I	Q	I	K	H		
II	Dw2-B	Dw2	R	W	P	R	D	Y	F	S	V	F	I	Q	A	V	V	Q	S	K	P	Q	S	L	G	M	D	T	R	V	R	Q		
	DHO-8	Dw12	R	W	P	R	D	Y	F	S	V	F	I	Q	A	V	G	Q	S	K	P	Q	S	L	G	M	N	T	R	V	R	Q		
	MN2-61	MN2	R	W	P	R	D	Y	F	S	V	Y	F	D	R	V	G	Q	S	K	P	Q	S	L	G	M	N	T	R	V	R	Q		

The table lists differences in the amino-acid sequences of six DR β -chains from three different HLA-DR2-positive individuals. Positions not shown in the table are identical in all six DR β -chains (see ref. 18 for a complete listing of shared amino-acid residues). The Dw2-A and Dw2-B sequences are from protein sequencing of two DR β -chains of a DR2-Dw2 homozygous cell line¹⁸. The other four sequences are deduced from cDNA clones obtained from cDNA libraries constructed in our laboratory. These four cDNAs encode DR β molecules as revealed by restriction mapping (especially characteristic internal *Pst*I sites) and comparison with available DR β sequences^{6,7}. DHO (DR2-Dw12) is a homozygous typing cell (HTC) line¹⁷. The MN2-2 and MN2-61 sequences are from an individual typed as DR2-MN2/DR4-Dw4. MN2-2 and MN2-61 are considered to be DR β sequences from the MN2 haplotype because: (1) their sequences are different from the two published DR β genes of the DR4-Dw4 haplotype^{35,41}; (2) they have greater homology to other DR2-derived DR β sequences than to DR4 DR β sequences; (3) we have sequenced another DR β cDNA from the same cell line and found it to be identical to other DR4-Dw4 DR β sequences^{35,41} (S.W. *et al.*, unpublished observation). cDNA was synthesized using oligo(dT) to prime the first strand and nascent messenger RNA was nicked with RNase H to prime the second strand synthesis²². cDNA was 'blunt-ended' with DNA polymerase and tailed with dCTP using terminal deoxynucleotidyl transferase. cDNA was annealed with *Pst*I-cut, dG-tailed single-stranded plasmid vector pUC119 (ref. 42) and introduced into *E. coli* strain MV1193. Each library, which contained about 50,000 colonies, was screened with a 790-bp *Scl*I/*Hind*III fragment of a DR β cDNA clone⁴³ labelled with ³²P by nick translation. This probe contains almost the entire coding region from this clone and under the hybridization conditions used, reacts most strongly with DR β cDNA but also reacts weakly with DQ β and DP β cDNAs. The inserts were sequenced directly on pUC119 or truncated subclones were generated by the method of Dale *et al.*⁴⁴. The second orientation was sequenced from inserts subcloned into plasmid vector pUC118 or M13 mp18. Sequencing was done in both orientations using the dideoxy chain termination method⁴⁵ with the M13 universal sequencing primer⁴⁶ or oligonucleotide primers corresponding to conserved DR β sequences. The DHO-7 clone contains a truncated insert. The available sequence of DHO-7 starts at amino-acid⁴⁵. Amino-acid residues of interest are boxed. Residues not available for comparison are marked with a dash. CP refers to the connecting peptide, TM to the transmembrane region and CY to the cytoplasmic tail of the DR β molecule. The total length of the DR β -chain, not including the signal peptide, comprises 237 amino acids.

Table 2 χ -Like sequences in the immunoglobulin-MHC supergene family

Species	Gene	Altered nucleotide/consecutive nucleotides*	χ -Like sequence	Refs
Murine	MHC-class II A ^{bm12}	3/14	GCTGGGG	11, 12
Murine	MHC-class II K ^{bm1}	7/13	GCTGGTG	9, 10
Human	MHC-class II DR3-DR β 1	7/34	GCTGGGG	14
Human	MHC-class II DR2-DR β	7/18	GCTGGGG	This paper
Human	MHC-class I B27k	4/14	GCTGGTG	13
Murine	IgD V gene	15/164	GCCTGGGG(CTGA)GCTGGTG(AA)GCCTGGGG	32

The χ sequence is GCTGGTGG or its complement or its duplex²⁶. The χ -like sequence for the immunoglobulin switch region is GCTGGTG(G) (ref. 28), for Ti-plasmid GCTGGTGG and GCTGTGG (ref. 29), for a core sequence in human 'minisatellite' DNAs GGAGGTGGGCAGGA₃G (ref. 30), and for murine class II E β gene (GGCA)₂₅₋₃₇ (L. Hood, personal communication).

* The number of altered nucleotides/the number of consecutive nucleotides from the first to last alteration.

of shared amino-acid residues.) The six sequences can be classified unambiguously into two groups by sequence homology; they presumably represent the two expressed DR β molecules in the DR2 haplotype^{19,20}. The group I DR β molecules are apparently more polymorphic than the group II DR β molecules. Of greatest importance are amino acids 67–71 (Table 1). These five residues are shared by MN2-2 in group I and two sequences in group II (Dw2-B and DHO-8); also, MN2-61 in group II shares these five residues with two sequences in group I (Dw2-A and DHO-7). Corresponding cDNA nucleotide sequences of these DR β molecules (Fig. 1) show that DHO-7 (group I) and MN2-61 (group II) share all 18 nucleotides coding for residues 67–72, as do DHO-8 (group II) and MN2-2 (group I). These two 18-nucleotide sequences differ at 7 positions, resulting in 3 amino-acid substitutions out of 6. It is extremely unlikely that seven nucleotide differences are due simply to the accumulation of sequential point mutations²¹, especially as the third base of each codon is the same for each amino acid. The simplest explanation of this finding is that there has been reciprocal genetic exchange of this segment between the two non-allelic expressed DR β genes.

The non-reciprocal gene conversion events implicated in generating the A^{bm12} allele (murine)^{11,12}, DR3 haplotype (human)¹⁴ and various DR4-Dw subtypes^{14,22,23} involve the same third

hypervariable region in the first domain of the class II gene. This hypervariable region apparently specifies an epitope for alloreactivity^{11,22–24} and restricted recognition²⁵. The finding that this functionally important segment of the class II gene is frequently involved in gene conversion-like events prompted us to look for a unique sequence which may promote genetic recombination^{15,16,26,27}. These include the χ sequence in λ phage and *Escherichia coli* (5'-GCTGGTGG-3' or its complement or its duplex)²⁶ and the repeated T-G and C-G dinucleotides implicated in a gene conversion event in the human globin genes²⁷.

χ -Like sequences have been recognized in DNA which undergo rearrangement or recombination (refs 28–30 and L. Hood, personal communication) (Table 2). A χ -like sequence (5'-GCTGGGG-3') is found 36 base pairs (bp) upstream from the 18-bp 'interconverted' hypervariable segment within a region of complete sequence homology from amino-acid residue 48 to 66 among all four DR β cDNAs that we have sequenced (from the second exon) (Fig. 1). The amino-acid sequences for Dw2-A and Dw2-B (ref. 18) (Table 1) are consistent with such a nucleotide sequence.

There are two other χ -like sequences in the DR2 β cDNAs, one near amino acid 1 (from the first exon) (Fig. 1) and another near residue 140 (5'-GCTGGGG-3' for both MN2-2 and DHO-7; and 5'-GCTGGGA-3' for both MN2-61 and DHO-8; these

hypothesize that the intergenic exchange was initiated by the χ -like sequence and involved the two non-allelic DR β I and DR β III genes on the same chromatid looping back on itself in a single genetic event with the reciprocal exchange of gene segments^{36,37}. Our model would be equally applicable if exchange occurred between sister chromatids or between chromosomes that have a high degree of sequence homology and carry the two 18-nucleotide sequences encoding residues 67–72 (Fig. 2). However, we do not exclude the possibility that reciprocal intergenic exchange resulted from two temporally independent non-reciprocal gene conversion events, or that double crossing-over can account for our observation, given the fact that 24–74 bp of complete sequence homology are sufficient for genetic recombination (segments c and e in Figs 1 and 2)^{38–40}, although this is less likely given interference between closely linked genetic markers in crossing over.

Gene conversion and reciprocal intergenic exchanges in MHC genes frequently involve hypervariable regions specifying functional epitopes and may allow the re-shuffling of functionally important epitopes to increase the combinatorial usage of a limited number of hypervariable regions and to create novel H-2 and HLA haplotypes.

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Predominant use of a V_α gene segment in mouse T-cell receptors for cytochrome c

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The T-cell receptor is a cell surface heterodimer consisting of an α and a β chain that binds foreign antigen in the context of a cell surface molecule encoded by the major histocompatibility complex (MHC), thus restricting the T-cell response to the surface of antigen presenting cells^{1–4}. The variable (V) domain of the receptor binds antigen and MHC molecules and is composed of distinct regions encoded by separate gene elements—variable (V_α and V_β), diversity (D_β) and joining (J_α and J_β)—rearranged and joined during T-cell differentiation to generate contiguous V_α and V_β genes^{5–11}. T-helper cells, which facilitate T and B cell responses, bind antigen in the context of a class II MHC molecule. The helper T-cell response to cytochrome c in mice is a well-defined model for studying the T-cell response to restricted antigen and MHC determinants. Only mice expressing certain class II molecules can respond to this antigen ($E_\alpha^k E_\beta^k$, $E_\alpha^k E_\beta^b$, $E_\alpha^d E_\beta^d$ and $E_\alpha^s E_\beta^s$)^{12–14}. Most T cells appear to recognize the C-terminal peptide of cytochrome c (residues 81–104 in pigeon cytochrome c)^{15–18}. We have raised helper T cells to pigeon cytochrome c or its C-terminal peptide analogues in four different MHC congenic strains of mice encoding each of the four responding class II molecules. We have isolated and sequenced seven V_α genes and six V_β genes and analysed seven additional helper T cells by Northern blot to compare the structure of the V_α and V_β gene segments with their antigen and MHC specificities. We have added five examples taken from the literature^{19–22}. These data show that a single V_α gene segment is responsible for a large part of the response of mice to cytochrome c but there is no simple correlation of MHC restriction with gene segment use.

The junctional nucleotide sequences for the V_α and V_β genes are given in Figs 1 and 2, respectively, and data for all the T cells examined are summarized in Table 1. These data, with functional studies to be discussed subsequently, demonstrate several interesting points.

The V_α 11.1 gene segment is the one most often used in the mouse helper T-cell response to cytochrome c (14 out of 19 T_H cells). The closely related V_α 11.2 gene segment (eight nucleotide substitutions out of 312) is used in the 15th T_H cell. The other predominant gene segments are used far less frequently than V_α 11.1 (J_α 84, 6 of 12; V_β 3, 7 of 19; J_β 1.2, 7 of 12). Some of

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Table 1 Gene segment usage by T-cell receptors recognizing pigeon cytochrome *c*

T cells*	Haplotype	α chain		β chain	
		V ⁺	J	V	J
AN6.2	E α^k E β^k	11.1‡	84	3	1.2
V11.5	E α^k E β^b	11.1	84	3	1.2
C.F6	E α^k E β^b	11.1	84	3	1.2
AN14.4	E α^k E β^b	11.1	14.4	3	1.2
5C.C7	E α^k E β^b	11.1	C7	3	1.2
BC15.1	E α^k E β^b	11.1	84	Y#	ND
BC37.5	E α^k E β^b	11.1	37	14#	ND
B10	E α^k E β^b	11.1	84	B10	2.1
4.C3	E α^k E β^b	11.1	84	B10	2.1
V1.9.2	E α^k E β^b	11.1	28	1	1.1
AR8.1	E α^k E β^b	11.1§	ND	Y#	ND
V15.4	E α^k E β^b	11§	ND	8.3	1.4
AP10.10	E α^k E β^b	11§	ND	8#	ND
AR10.1	E α^k E β^b	11§	ND	Y#	ND
2B4	E α^k E β^b	11.2	2B4	3	2.5
AP11.2	E α^k E β^b	4.3	11.2	3	1.2
AP15.2	E α^k E β^b	X§	ND	1	1.2
AN4.4	E α^k E β^b	X§	ND	8#	ND
AP7.10	E α^k E β^b	X§	ND	8#	ND

*C.F6, 5C.C7, B10 and 4.C3 are cloned helper T-cell lines induced in B10.A mice immunized with pigeon cytochrome *c*²². The rest of the cells are T-helper hybridomas generated as described⁴⁶. 2B4 has been reported previously^{19,20}. Hybridomas were made by fusing BW5147 lymphoma cells with cytochrome *c*-specific T-cell lines induced in four different congenic mouse strains: B10.A (E α^k E β^k ; AN and BC hybridomas), B10.SM (E α^k E β^b ; AR hybridomas), B10.A (5R) (E α^k E β^b ; V hybridomas) and B10.S (9R) (E α^k E β^b ; AP hybridomas). The mice were immunized with either pigeon cytochrome *c* (2B4 and BC hybridomas) or synthetic C-terminal homologous peptides to cytochrome *c*: Sp (AN hybridomas) or DASp (V, AP, AR hybridomas)⁴⁶. B10.A and B10.A(5R) hybridomas respond to antigen presented on E α^k E β^b or E α^k E β^b antigen presenting cells (APC) and B10.S(9R) and B10.SM hybridomas respond to antigen on E α^k E β^b or E α^k E β^b APC¹².

† Gene segment use was determined by DNA sequence analysis (for methods see legend of Fig. 1) unless otherwise indicated. Nomenclature is essentially as described²¹⁻²³ with the exception of J α 14.4 and J α 11.2 which have not been reported previously (for sequence see legend of Fig. 1). The two distinct members of the V α 11 subfamily are denoted V α 11.1 and V α 11.2; these two members have been previously referred to as V α 2B4.2 and V α 2B4.1, respectively²². They differ by only eight nucleotides in the coding region and nine nucleotides in the 5' flanking region (ref. 22 and data not shown). The V α gene of hybridoma AP11.2 is denoted V α 4.3 to distinguish it from the two published V α sequences belonging to the V α 4 subfamily²³. V β 3 has also been referred to as V β 2B4²².

‡ Boxes indicate predominant gene segment use.

§ Determined by Northern blotting using gene segments from V1.9.2 and AP11.2 as probes. Use of V α 11.1 by AR8.1 was determined by additional Southern genomic blotting using a V α 11 flanking region probe. V α other than V α 4 and V α 11 are denoted 'X'.

Determined by Northern blotting using probes from the V β 2,3,4,5,8 and 14 subfamilies. V β other than those used as probes denoted by 'Y'.

this apparent degeneracy may reflect the diversity of antigens and/or MHC molecules used in immunization²² (see Table 1), but the V α 11.1 protein sequence is clearly highly favoured in the construction of a specific antigen binding site for cytochrome *c*.

Some nucleotides in the V α junctional region are not encoded by either germline V α or J α gene segments (Fig. 1). Comparison of the known germline J α 84 and J α 28 sequences with those given in Fig. 1 allows us to determine where these J α sequences

	V	N or D	J
B10	V α 11.1 — TGTGCTGCTGAG		GCAACTTCAAGTG — J α 84
AN6.2	V α 11.1 —	AG *	— J α 84
V11.5	V α 11.1 —	AG *	— J α 84
C.F6	V α 11.1 —	C *	— J α 84
BC15.1	V α 11.1 —		— J α 84
4.C3	V α 11.1 —	T *	— J α 84
AN14.4	V α 11.1 —	G †	— J α 14.4
5C.C7	V α 11.1 —	G †	— J α 7
BC37.5	V α 11.1 —	TT †	— J α 37
V1.9.2	V α 11.1 —	CAGG *	— J α 28
2B4	V α 11.2 —	CTGAGG †	— J α 2B4
AP11.2	V α 4.3 —	CTGAGG †	— J α 11.2

Fig. 1 V α junctional nucleotide sequences of T-cell receptors recognizing cytochrome *c*. Left, names of T-helper hybridomas and clones analysed. Sequences of the C.F6, B10, 4.C3, 5C.C7 T-cell clones²² and of the 2B4 hybridoma^{19,20} have been reported previously and are included for comparison. Variable (V) and joining (J) gene segment sequences up- and downstream of those shown were found to be identical to previously published sequences¹⁹⁻²³ with the exception of V α 4.3, J α 14.4 and J α 11.2, which have not been reported previously. The V α 4.3 gene segment belongs to the V α 4 subfamily (at least 75% homologous to two previously reported V α 4 sequences²³) and differs from the V α 11.1 gene segment of hybridoma AN6.2 by 80% at the protein level and 61% at the DNA level. The V α 4.3 sequence is:

GGCTTCGTGGCTGTGACTTCTCATCTTGAAGGAC
CCACGGAGATTCCGTGACTCAAACAGAAGGCCAGTGA
CCGTCTCAGAAAGCGAGTCCCTGATAATAAATTGCACGT
ATTACAGCCACAAGCATAGTACTACCTAATCTTTTCTGG
TATGTTCCGATATCCTGGAGAAGGCTACAACTCTCC
TGAAAGTCATTACGGCTGGCCAGAAGGGAAGCAGCA
GAGGGTTTGAAGCCACATACAATAAAGAAACCACCTC
CTTCCACTTGCAGAAAGCCTCAGTGCAAGAGTCAGA
CTCGGCTGTGACTACTGTGCT.

The J α 14.4 sequence is:

CTTCTAATTACAACGTGCTTTACTTCCGATCTGGCACC
AACTCACTGTAGAGCCAAAGTAAGTAAGTTATTGTTG
TTATCCCACTTATGCTAAAT.

The J α 11.2 sequence is:

CTGGAGGCTATAAAGTGGTCTTTGGAAGTGGGACTCGA
TTGCTGGTAAGCCCTG.

V and J region boundaries were determined by comparison to the germline sequences of V α 11.1 (ref. 22), J α 84 (ref. 47) and J α 28 (A.W., unpublished data) and to the TA37 cDNA sequence (J α 37, ref. 23). Nucleotides which could not be assigned to either V or J gene segments are designated 'N' or 'D' and represent either additional non-germline sequences (*) or could not be evaluated because germline sequences are not known (+). Dots indicate identity to the B10 sequence.

Methods. The V α genes of T-cell clones C.F6, B10, 4.C3 and 5C.C7 (ref. 22) and of hybridoma 2B4 (refs 19, 20) were isolated from complementary DNA libraries as described. The V α genes of hybridomas V1.9.2, V11.5 and AP11.2 were isolated from λ gt10 cDNA libraries and subcloned into m13mp8. The rest of the hybridomas were isolated from limited genomic libraries constructed in λ L47.1 or EMBL3 vectors and were subcloned into M13 mp18 and/or 19. Both strands of DNA were sequenced using the dideoxy chain termination method⁴⁸ with the M13 universal primer and a C α -specific oligonucleotide (CA3) primer for the cDNA clones, and specific oligonucleotide primers for the genomic clones.

end in the 5' direction. Although the germline sequence of V α 11.1 is not known, clearly four of the five different sequences denoted by asterisks in Fig. 1 must represent extra nucleotides (the fifth could be the germline V α 11.1 sequence). Two explanations are possible. First, the extra nucleotides could represent contributions from D α gene segments. This explanation seems unlikely because the number of added nucleotides is small (1-4) and no other evidence for D α gene segments has yet been reported²³. The second possibility is that N-region diversification, the addition of random nucleotides in the junctional region to either of the joined gene segments^{24,25}, may occur. It has been proposed that N-region diversification is caused by the enzyme terminal deoxynucleotidyltransferase (TdT)²⁶, which is present in T cells

Fig. 2. V_β junctional nucleotide sequences of T-cell receptors recognizing cytochrome *c*. Sequences of the C.F6, B10, 4.C3, 5C.C7 T-cell clones²² and 2B4 (refs 19, 20) and V1.9.2 (refs 19, 21) hybridomas have been reported previously and are included for comparison. Only nucleotides in and around the diversity (*D*) gene segment are shown; *V* and *J* sequences both up- and downstream of this region were found to be identical to those published previously^{5,19,21,49}. *V*, *D* and *J* region boundaries were determined by comparison to known germline sequences ($V_\beta 3$ (ref. 19); $D_\beta 1.1$ (refs 31, 32); $J_\beta 1.1$, 1.2 and 1.4 (ref. 5); $J_\beta 2.1$ and 2.5 (refs 5, 19)) and to the cDNA sequences of BW5147 ($V_\beta 1$, ref. 21), 86T1 ($V_\beta 1$, ref. 49) and TB23 ($V_\beta 8.3$, ref. 21). Dots, identity to the AP11.2 sequence or to the $D_\beta 1.1$ sequence. The T nucleotide in the *D* segment of 2B4 (*) corresponds to the T at position 6 of $D_\beta 2.1$ (GGGACTGGGGGGGC, refs 31, 32).

Methods. The V_β genes of T-cell clones C.F6, B10, 4.C3 and 5C.C7 (ref. 22) and hybridomas 2B4 (refs 19, 20) and V1.9.2 (refs 19, 21) were isolated from cDNA libraries as described. The other V_β genes were isolated from limited genomic libraries constructed in the λ L47.1, EMBL3, λ gt10lac5 or λ 590 vectors and sequenced in the same manner as the V_α genes.

<i>V</i>		<i>D</i>	<i>J</i>
AP11.2	$V_\beta 3$	GGGACAGGGGGC	
AN6.2	$V_\beta 3$	A...CG	CAAACTCCGACTACA — $J_\beta 1.2$
V11.5	$V_\beta 3$	TCGG...CGG	— $J_\beta 1.2$
C.F6	$V_\beta 3$	A...TG	— $J_\beta 1.2$
AN14.4	$V_\beta 3$	AACGC	— $J_\beta 1.2$
5C.C7	$V_\beta 3$	A...ATG	— $J_\beta 1.2$
AP15.2	$V_\beta 1$	TCGT...G	— $J_\beta 1.2$
2B4	$V_\beta 3$	A...T...AG *	CC AGA AC C GT — $J_\beta 2.5$
B10	$V_\beta B10$	CC...CA	AACT TG T GC GT — $J_\beta 2.1$
4.C3	$V_\beta B10$	TTA...CAGT	CT TG T GC GT — $J_\beta 2.1$
V1.9.2	$V_\beta 1$	A...G	— $J_\beta 1.1$
V15.4	$V_\beta 8.3$	GATGCG...AA	TTT C...GAAAGA TAT — $J_\beta 1.4$

during early development at the time of rearrangement of the T-cell receptor genes^{27–30}. In contrast, N-region diversification is never seen in immunoglobulin light chains^{24,25}, which also lack *D* gene segments, presumably because TdT is not present in these cells when the gene segments are rearranged. TdT shows a clear preference for the addition of G residues²¹ and three out of five of the asterisked sequences in Fig. 1 contain G as their sole or predominant nucleotide. Although no conclusions can be reached about the five sequences denoted in Fig. 1 as '+' because we do not have the corresponding germline V_α and/or J_α gene segment sequences, N-region diversification may add up to 15 bases in the joining process^{24,25}. The two remaining unmarked sequences in Fig. 1 can be totally accounted for on the basis of germline sequence and thus represent 'error-free' *V*–*J* joining. This agrees with the suggestion¹⁹ that the use of N-region diversification or the joining of a D_α gene segment is not required for productive recombination.

It is striking that all the rearranged V_α and V_β genes are different in sequence except for V_α AN6.2 and V_α V11.5. This enormous variability arises through: (1) use of a multiplicity of distinct gene segments; (2) joining these gene segments in different combinations and (3) diversity of junctional regions arising because of N-region diversification or the flexibility in the regions at which the gene segments are joined. There is extensive junctional flexibility in the V_α and V_β genes and N-region diversification in the V_β genes^{31,32} (Figs 1 and 2). This variability emphasizes the fact that striking diversity in the third hypervariable region is compatible with T-cell receptor specificity for particular antigen-MHC determinants.

There is no simple correlation between class II MHC restriction and T-cell receptor gene segment usage (Table 1). For example, in helper T cells raised in $E^k E^k$ mice, the most frequently studied haplotype, three different V_α (11.1, 11.2, and X), three different J_α (84, 14.4 and 2B4), five different V_β (3, 14, B10, 8 and Y) and three different J_β (1.2, 2.1 and 2.5) gene segments are used. Moreover, the V_α 11.1 gene segment is used by all four haplotypes studied, and the V_β 8 and V_β 3 gene segments are present in three of these four haplotypes. The enormous diversity observed in the junctional regions of α and β chains suggests that there are many different ways to select the T-cell receptor molecule for restriction to a particular MHC molecule.

Analysis of the ten V_α 11.1 gene segments reveals no nucleotide differences (Fig. 1 and data not shown). Since all ten T-helper hybridomas are functional and presumably represent mature T cells, we conclude that somatic hypermutation occurs infrequently, if at all, in the α genes of T_H cells specific for cytochrome *c*. Similar conclusions have been reached for the β genes of the T-cell receptor^{9,21,33} and our data are consistent

with this interpretation in that eleven V_β gene segments from three subfamilies are identical in their coding region nucleotide sequences to their subfamily members, except for one mutation at position 95 of the V11.5 V_β gene segment (Fig. 2). Somatic hypermutation in B cells, essential for affinity maturation³⁴, appears to occur relatively late in B-cell differentiation^{35–37}. Our T cells were hybridomas generated from purified lymph node cells that were stimulated once *in vivo* and a week later placed in tissue culture with antigen for three days before fusion. Thus the possibility that somatic hypermutation has not occurred because our T cells are analogous to primary B cells or because

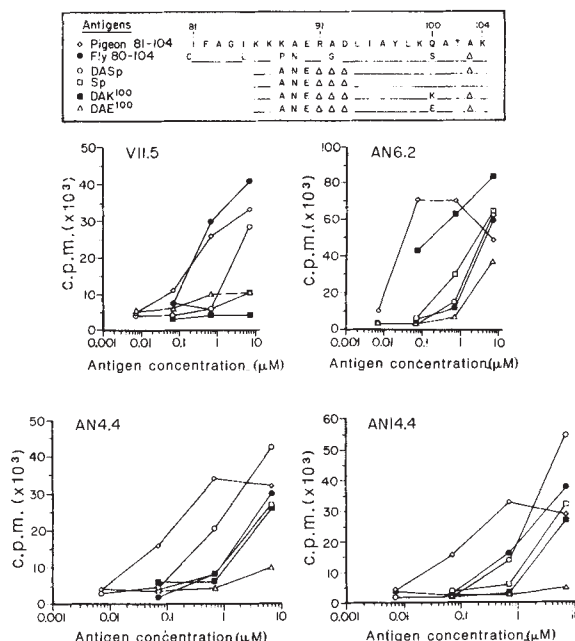


Fig. 3 Fine specificity of cytochrome *c* peptide-specific T hybridomas. Hybridoma cells were cultured with varying concentrations of pigeon cytochrome *c* and B10.A ($E^k E^k$) spleen cells as a source of antigen presenting cells. After 40 h of culture, supernatants were transferred to the IL-2-dependent cell line HT-2. ³H-thymidine was added to the secondary culture 16 h later. Antigens were C-terminal peptides of pigeon or fly cytochrome *c*, or synthetic derivatives of the pigeon peptide^{46,50}. Note that an N-terminal cysteine has been added to the fly sequence. A dash denotes identity; Δ denotes a deletion (compared with the pigeon sequence).

of the antigen excess condition *in vitro* cannot be excluded. However, the data here and data from 27 human V_β sequences from peripheral blood³⁸ are consistent with the supposition that somatic hypermutation does not play an important role in generating the diversity of the T-cell receptor repertoire. Hence, affinity maturation may not play any significant role in the T-cell recognition of antigen-MHC and the affinity of T-cell receptors for their cognate antigen may be generally lower than that of antibodies for their cognate antigen.

The V_α and V_β chains from hybridomas AN6.2 and V11.5 are identical except for five amino-acid differences in the V_β hypervariable region formed by $V-D-J$ joining (Fig. 2). We studied the antigenic fine specificity of these hybridomas to six different related cytochrome *c* peptides (Fig. 3). These two T cells responded differently to the panel of peptides. For example, hybridoma V11.5 did not respond to the peptide DAK¹⁰⁰ even at high levels of antigen concentration, whereas hybridoma AN6.2 reacted strongly with this peptide. Although no MHC fine specificity data is available for these two T-cell clones, we know that both respond equally well to antigen presented on cells bearing $E^k_\alpha E^k_\beta$ or $E^k_\alpha E^b_\beta$ MHC molecules and not to cells bearing $E^k_\alpha E^s_\beta$ or $E^s_\alpha E^b_\beta$ molecules. We thus conclude that changes limited to the junctional region of the V_β sequence may alter the antigenic fine specificity of individual T cells without altering their predominant MHC specificity. As this region seems to correspond to the third hypervariable segment of immunoglobulin V region sequences^{21,33,39,40}, we conclude that this region is very probably part of the antigen binding site—consistent with the supposition that the V regions of T-cell receptors and immunoglobulins fold in a similar manner.

Further fine specificity studies on two other hybridomas using six different cytochrome *c* peptides resulted in a striking observation. Hybridomas AN4.4 and AN14.4, which do not share the same pair of V_α and V_β gene segments, show nearly identical profiles of antigen fine specificity within the panel of peptides tested (Fig. 3; pigeon cytochrome *c* > DASp peptide > fly cytochrome *c* > Sp peptide > DAK¹⁰⁰ peptide > DAE¹⁰⁰ peptide). The fact that two hybridomas which are similar in their antigen fine specificity and predominantly restricted to $E^k_\alpha E^k_\beta$ do not share the same pair of V_α and V_β gene segments suggest that a combination of different V_α and V_β regions can form very similar antigen-MHC binding pockets, again consistent with the idea that affinity for cognate antigen may be relatively low.

The V_α -restricted nature of the cytochrome *c* T-cell response raises interesting clinical possibilities. Several animal models of human autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and Hashimoto's thyroiditis provide strong evidence for the pathogenic involvement of T cells in the development of these autoimmune disorders⁴¹⁻⁴⁵. These models also indicate that T-cell responses responsible for these autoimmune diseases are directed against restricted antigen determinants. These situations may be similar to the T-cell response against cytochrome *c* and raise the possibility that these autoimmune diseases may be associated with predominant usage of a particular T-cell receptor V_α (or V_β) gene segment. Antibodies to these V gene segments might then have useful diagnostic or therapeutic possibilities.

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High frequency of unequal recombination in pseudoautosomal region shown by proviral insertion in transgenic mouse

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The mammalian X and Y chromosomes, in contrast to the autosomes, pair during male meiosis only near the telomeres. Alleles localized in this region can undergo reciprocal exchange during meiosis¹⁻⁴. Because such sequences do not show strict sex-linked inheritance, they have been termed pseudoautosomal⁵. In man, several DNA sequences have been described which show pseudoautosomal transmission and which are localized in the pairing region at the ends of the short arms of both the X and Y chromosomes (refs 6-9, and D. Page, unpublished results). We now show that the transgenic mouse strain, Mov-15, contains a single Moloney murine leukaemia virus (M-MuLV) genome in its

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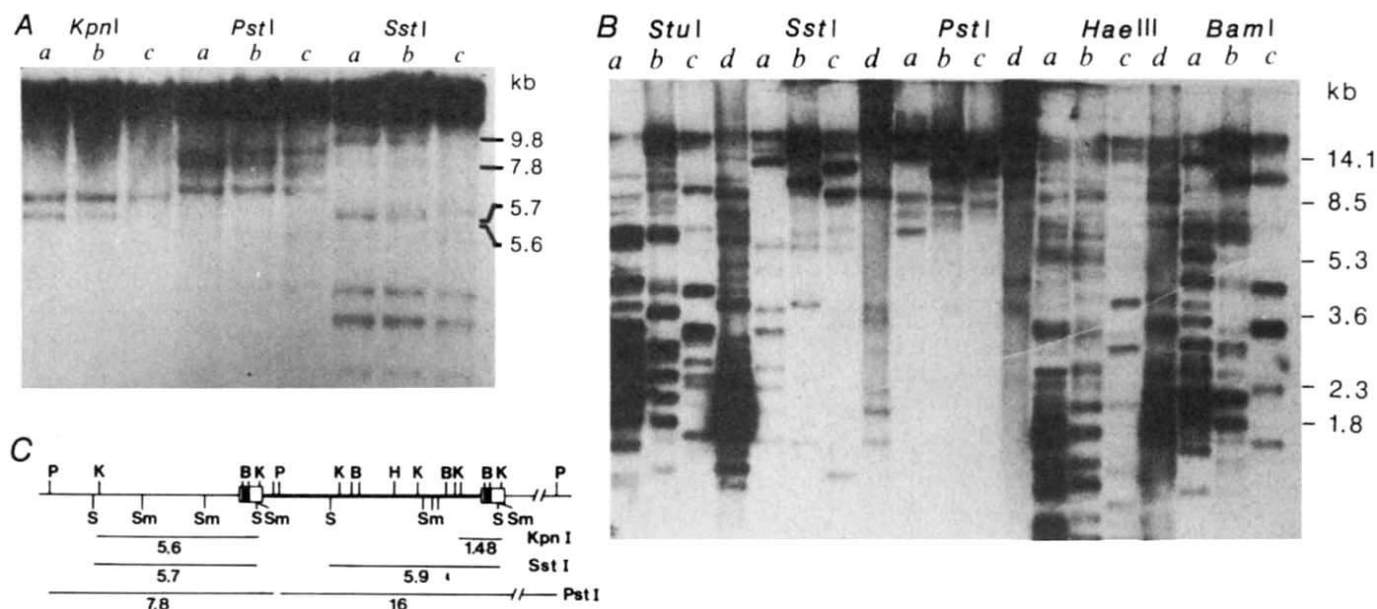


Fig. 1 Restriction map and cloning of the Mov-15 locus. **A**, Isolation of mouse DNA sequences flanking the Mov-15 provirus. A recombinant plasmid, pMov-15/1, containing sequences flanking the provirus, was hybridized to DNAs from homozygous Mov-15 males (lane a), heterozygous Mov-15 males (lane b) and BALB/c males (lane c) digested with the indicated restriction enzymes. **B**, DNA polymorphism at the Mov-15 locus. DNAs from BALB/c (lanes a), C57BL/6 (lanes b), 129/J (lanes c), and SWR/J (lanes d) mice were digested with the restriction enzymes shown and analysed by Southern blot analysis as described in **A**, using pMov-15/1 as a probe. **C**, Restriction map of the proviral region. White boxes, LTRs; black boxes, the bacterial *supF* gene; black bar, the proviral genome and the line left and right from the provirus, the flanking mouse sequences. K, P, H, S and Sm denote cleavage sites for *KpnI*, *PstI*, *HaeIII*, *SstI* and *SmaI*, respectively. The map was deduced from restriction enzyme analysis of cellular DNA from homozygous Mov-15 mice. The lengths of internal and flanking fragments hybridizing with the bacterial suppressor gene are indicated in kb below the figure.

Methods. DNAs were purified, digested with the enzyme indicated, separated on a 0.8% gel and transferred to Gene Screen Plus membranes (NEN)¹⁰. The filters were hybridized to probes labelled to high specific activity by random oligonucleotide priming²⁶. Filters were washed in 0.1×SSC, 0.1% SDS, at 68 °C. As predicted from the map in **C**, hybridization of the pMov-15/1 probe revealed the presence of a 5.6 kb *KpnI* fragment, a 7.8 kb *PstI* fragment and a 5.7 kb *SstI* fragment in Mov-15 mice (lanes a and b). These fragments were not seen in DNAs from wild-type mice (lane c) and the results therefore confirm that pMov-15/1 represents cellular sequences flanking the provirus. The 9.8 kb *SstI* fragment found exclusively in DNAs from Mov-15 mice (lanes a and b) most probably represents a flanking cellular fragment from the 3' end of the provirus which hybridized with pMov-15/1 due to the repetitive nature of the probe. As expected, the intensities of the Mov-15 specific fragments in homozygous animals were about twice that seen in DNA from heterozygous animals (compare lanes a and b). The 5.7 kb *SstI* fragment had been cloned by extracting DNA from the liver of a homozygous Mov-15 female mouse, digesting it with *SstI* and fractionating it in a sucrose gradient. Fractions containing the Mov-15 specific *SstI* fragments were identified by hybridization with the *supF* gene and ligated to *SstI*-cleaved λ gtWES arms. After *in vitro* packaging, recombinant phage particles were titred on the *sup*⁺ host MC 1061 p3¹². About 1.5×10⁶ plaque-forming units were then plated on the *sup*⁺ host MC 1061 p3¹². Eight plaques were obtained, five of which contained recombinant phages with the 5.7 kb flanking *SstI* fragment. The remaining three plaques contained recombinant phages with the internal 5.9 kb *SstI* fragment. This internal fragment was distinguished from the flanking *SstI* fragment on the basis of an internal *HindIII* site at nucleotide 4894 (ref. 27). A recombinant λ phage containing the flanking sequences was cleaved with *SstI*, the 5.7 kb insert isolated by preparative gel electrophoresis, further digested with *BamHI* to remove most of the LTR, and the *BamHI*-*SstI* fragment comprising about 90 bp of the LTR plus the flanking mouse sequences was subcloned in pUC 19. The resulting plasmid was designated pMov-15/1.

germline, and genetic evidence indicates that the provirus is integrated into the pseudoautosomal region of the sex chromosome. Proviral copies are lost or gained in 7% of male meioses in this strain, and mouse sequences flanking the provirus are tandemly repeated and highly variable. We conclude that unequal recombination events occur with high frequency in the pairing region, possibly because of the presence of repeated sequences.

The derivation of the Mov-15 strain from M-MuLV^{sup}-infected preimplantation BALB/c mouse embryos has been described previously¹⁰. Since all Mov-15 animals develop viraemia, the provirus serves as a dominant marker whose expression can be monitored by radioimmunoassay of serum samples¹¹. Animals carrying the provirus were also identified by DNA blot analysis, using as a probe the bacterial suppressor gene present in the long terminal repeats (LTRs)¹². When two Mov-15 males of the N1 generation (obtained from breeding founder male A, ref. 10) were bred with wild-type BALB/c females, about 90% of male offspring and only 10% of female offspring carried the Mov-15 provirus (Table 1). This partial sex linkage suggested that the provirus in these males was located on the Y chromosome and was transferred to the X chromosome in about 10%

of the meioses, resulting in 10% viraemic daughters. To test this hypothesis, viraemic N2 females were bred with wild-type BALB/c males. As shown in Table 1, the Mov-15 provirus was transmitted equally to sons and daughters of the N3 generation. When viraemic males of the N3 generation which had received the provirus from their mother were bred with wild-type BALB/c females, partial X-linked transmission of the virus was observed (85% of the N4 daughters and only 19% of the N4 sons carried the virus), as expected if the provirus was carried on the X chromosome of the N2 mothers. Viraemic sons of the N4 generation in turn transmitted the virus in a Y-linked mode to the next generation. The results clearly show that the Mov-15 provirus is transferred from the Y to the X chromosome and back from the X to the Y chromosome in 10–20% of all male meioses (Table 1). A gradient of sex linkage has been established within the human pseudoautosomal region, and the recombination frequency can be used to estimate the position of the locus within this region⁹. Thus, markers at the telomeres recombine with frequencies of 50%, and the recombination rate decreases for more proximal markers. If the same holds true for the mouse pseudoautosomal region, our data localize the Mov-15 proviral

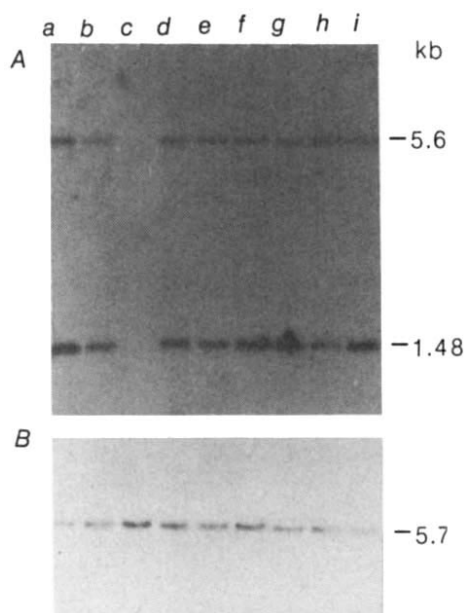


Fig. 2 Unequal recombination at the Mov-15 locus. A homozygous Mov-15 male was mated with a wild-type BALB/c female and embryos were isolated at day 14 of development. DNAs were purified and analysed by blot hybridization following digestion with *Kpn*I (Fig. 1 legend). A, Hybridization to π AN7, a plasmid containing the bacterial suppressor gene (gift of H. Huang). The flanking 5.6 kb fragment and the internal 1.48 kb fragment hybridizing to the probe (Fig. 1C) are indicated. Since viraemia might lead to the presence of additional proviral copies in the DNA samples, which would all contain the internal fragment, the intensity of the 5.6 kb flanking fragment, which is specific for the provirus inserted in the germ line, was used instead to estimate the number of Mov-15 proviral copies present. B, Hybridization with a *Bgl*II-*Hpa*I fragment containing part of the α 1(I) collagen gene²⁸; the intensity of the 5.7 kb hybridizing fragment serves as a control for the amount of DNA loaded in each lane.

genome to the proximal part of the X-Y pairing region. Three point crosses with distal markers such as the steroid sulphatase gene³ will allow us to test this hypothesis directly.

In order to analyse the organization of the Mov-15 locus, a probe corresponding to sequences flanking the provirus was isolated by cloning into λ gt WES using selection for the *supF* gene (ref. 12; see Fig. 1A legend). This probe, designated pMov-15/1, hybridized to differently-sized fragments in virus-positive and virus-negative animals, demonstrating that the cloned sequences represent cellular sequences flanking the Mov-15 provirus (Fig. 1A). In addition to the Mov-15 specific fragments, the probe hybridized with variable intensities to many other fragments of different sizes, even under stringent conditions of hybridization and washing. To analyse the flanking sequences further, the insert of pMov-15/1 was digested with *Sma*I and the three fragments produced (Fig. 1C) were sub-cloned and used as probes on Southern blots. Each fragment produced identical patterns of hybridization to those shown in Fig. 1A. Each fragment hybridized with all the others, confirming that the cellular sequences flanking the provirus are not only repeated many times in the genome, but are also internally repetitive. The repeat length unit is at the most 1.3 kilobases (kb), the length of the smallest fragment tested.

The flanking sequences were also used to probe DNAs from different mouse strains. Hybridization bands of different size and intensity were observed when DNAs from different mouse strains were compared (Fig. 1B). This polymorphism was not detected when males and females of the same inbred strain (BALB/c) were compared (data not shown). The sequences are

Table 1 Sex-chromosome-linked transmission of Mov-15 provirus

Parents		Offspring			
Inferred genotype	Backcross generation	Sex	Offspring with provirus/total tested	%	
F	M				
wt/wt	Y ^{Mov} /X ^{wt}	N2	M	116/130	89
	(N1)	F		19/191	10
X ^{Mov} /X ^{wt}	wt/wt	N3	M	31/57	54
	(N2)	F		33/51	64
wt/wt	Y ^{wt} /X ^{Mov}	N4	M	19/99	19
	(N3)	F		130/152	86
wt/wt	Y ^{Mov} /X ^{wt}	N5	M	43/49	88
	(N4)	F		14/70	20

All offspring from crosses of Mov-15 males with wild-type BALB/c females were analysed for the presence of virus in the serum by radioimmunoassay using antibodies against p30 (ref. 11). In addition, about 10% of the offspring were analyzed for the presence of proviral sequences by DNA blot hybridization. No discrepancies were observed when the results of both tests were compared. Since virus can be efficiently transmitted by milk from a viraemic mother to her offspring²⁴, all offspring of Mov-15 females with wild-type BALB/c males (N3) were analysed for the presence of the provirus by blot analysis of tail DNA samples. Among the offspring of the original founder animal (founder A, ref. 10), 3 out of 19 male offspring and 7 out of 27 female offspring contained the Mov-15 provirus. Because of the relatively small number of provirus-carrying offspring, a clear assignment of the provirus in the founder animal to the X or to the Y chromosome could not be made, but two of the three provirus-carrying males were found to have the provirus on the Y chromosome (lines 1 and 2). wt, wild-type; M, males; F, females.

specific for mouse and do not hybridize, even under low stringency, to DNAs from man, hamster, rat, pig and chicken (data not shown). Repetitive and highly polymorphic sequences have been found previously in the human pseudoautosomal region (refs 6-9, and D. Page, unpublished results) as well as on the mouse Y chromosome^{13,14}.

To derive animals homozygous for virus integration at the Mov-15 locus, heterozygous males carrying the provirus on their Y chromosome were crossed with heterozygous females. The genotypes of offspring were determined by Southern blot analysis using the autoradiographic intensity of the 5.6 kb Mov-15-specific *Kpn*I fragment (representing part of the LTR and 5' flanking sequences; Fig. 1C) to estimate the number of proviral copies present. Animals with two proviruses were classified as homozygous. As predicted, about equal numbers of homozygous and heterozygous sons and of heterozygous and wild-type daughters resulted from this cross; no wild-type sons or homozygous daughters were found (Table 2).

Table 2 M-MuLV genotype of offspring from parents both heterozygous at the Mov-15 locus

Cross: F(X ^{wt} /X ^{Mov}) × M(X ^{wt} /Y ^{Mov})*			
Genotype of offspring			
	Homozygous	Heterozygous	Wild type
M	18	22	0
F	0	14	18

The genotype of the offspring was determined by Southern blot analysis of tail DNA using the autoradiographic intensity of the 5.6 kb Mov-15 specific *Kpn*I fragment for estimating the number of M-MuLV^{sup} copies present (for details see legend to Fig. 2). wt, wild-type; M, males; F, females.

* The genotype X^{wt}/Y^{Mov} was assigned to the heterozygous males since they transmitted the Mov-15 provirus to about 90% of their male offspring and about 10% of their female offspring when mated with wild-type BALB/c females.

When males homozygous for virus integration were mated with wild-type BALB/c females, only 93% of the offspring were viraemic instead of the 100% expected for normal Mendelian transmission (Table 3). Southern blot analysis using the *supF* gene fragment or a representative M-MuLV as a probe confirmed that non-viraemic animals did not carry the provirus. As an example, Fig. 2 shows the analysis of embryos derived from one such cross. One of the embryos was negative for proviral sequences (lane c). No other fragments hybridizing with the *supF* probe were detected, suggesting that the absence of the provirus was not due to homologous recombination involving the LTRs, as such a mechanism of provirus excision should leave a single LTR at the integration site¹⁵. While the majority of the offspring carried a single Mov-15 provirus, some animals had acquired two copies. This was shown by comparing the intensities of the 5.6 kb *KpnI* fragments with a unique fragment as an internal standard (see lane a in Fig. 2A,B). Similar analyses of a larger number of embryos showed that about 7% had acquired two proviral copies (Table 3). The frequency of offspring which have lost or gained a proviral copy is therefore almost as high as the overall frequency of recombination between the X and Y chromosomes at the Mov-15 locus.

The results in Table 3 and Fig. 2 suggest that the region of the X and Y chromosomes into which the provirus has integrated can undergo unequal exchange during male meiosis, as indicated by the loss of the provirus on one sex chromosome (provirus negative offspring) and the gain of a second copy on the other sex chromosome (offspring with two proviral genomes). Blot hybridization analyses on pulse field gels demonstrated that restriction sites in cellular sequences flanking the provirus were identical over a distance of 70 kb (data not shown). Thus the recombination events involved large stretches of host sequences and the provirus was not reinserted into an unrelated part of the genome.

The most likely mechanism to explain the gain or the loss of proviral genomes is unequal crossing-over events between sister chromatids or between homologues either during gametogenesis or during meiosis. Such a mechanism would indeed predict that the two classes of offspring, either with no provirus or with two proviral copies, should arise at equal frequencies. To date, no direct genetic information on the occurrence and frequency of unequal crossing-over is available for mammals. The only mechanism known to occur in mammals which formally could result in unequal crossing-over is sister chromatid exchange. The frequency of sister chromatid exchange in spermatogonia has been determined to be less than two per cell for any chromosomes¹⁶, which is therefore orders of magnitude lower than the frequency we have observed for the unequal exchange of the Mov-15 provirus which occurs within a small chromosomal region. The most likely mechanism by which the Mov-15 provirus is deleted or duplicated is therefore unequal crossing-over during meiosis. Because each recombination event at the Mov-15 locus transfers at least 70 kb of sequences flanking the provirus from one sex chromosome to the other, our observations suggest that a high frequency of unequal recombination may be characteristic for the X-Y chromosome pairing region. In addition, the recombination frequencies we have determined in this work have been in male meiosis only. In the human pseudoautosomal region it has been estimated that the rate of recombination is tenfold higher in males than in females⁹. It will therefore be of interest to study recombination as well as unequal crossover frequencies in Mov-15 females.

It has been suggested that repetitive sequences can lead to unequal crossover events resulting in contraction or expansion of sequence families¹⁷. If such a mechanism is responsible for the unequal exchange observed at the Mov-15 locus, it may be possible to generate animals with larger numbers of proviral copies by successive breeding of offspring with newly-acquired Mov-15 proviruses and so allow comparison of unequal crossing-over frequencies in animals after 'expansion' or 'contraction'

Table 3 Transmission of Mov-15 provirus

Parents	Offspring carrying:	
	no provirus	two proviruses
F (wt/wt)	M: 3/76	
$\times$	F: 10/115	
M(X^{Mov}/Y^{Mov})	E: 7/109	8/109
	Total: 20/300 (6.7%)	8/109 (7.3%)

Males homozygous for virus integration at the Mov-15 locus were derived as described in Table 2 and mated to wild-type females. Offspring were tested for viraemia at the age of 4–6 weeks. Non-viraemic animals were analysed for the presence of the provirus by blot hybridization of tail DNAs. In addition, the same tail DNAs were analysed for the presence of the M-MuLV specific 5.9 kb and 2.6 kb *SstI* fragments as described²⁵. Embryo DNAs (isolated between day 12 and 14 of gestation) were analysed for the presence of the provirus by blot hybridization and the intensity of the Mov-15 specific 5.6 kb *KpnI* fragment on autoradiograms was used for estimating the number of M-MuLV copies present (for details see legend to Fig. 2). wt, wild-type; M, males; F, females; E, embryos.

of the Mov-15 pairing region. To date, unequal recombination has been directly documented in yeast^{18–20} and *Drosophila*²¹, but never in mammals, although it has been implicated in the evolution of multigene families²² and satellite DNAs^{17,23} and in the generation of autosomal²⁹ or sex-linked mutant loci¹. No information has been available until now on the frequency of such events during meiosis. Only the presence of the unique proviral marker has enabled us to measure the frequency of unequal recombination in a DNA region composed of repetitive sequences. Our results suggest that such exchanges may be frequent in regions where chromosomes synapse during meiosis.

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***In vivo* protein–DNA interactions in a glucocorticoid response element require the presence of the hormone**

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Transcriptional activation of gene expression by glucocorticoid hormones is mediated by the interaction of hormone–receptor complexes with specific DNA sequences called glucocorticoid responsive elements (GREs) (refs 1–3, see ref. 4 for review). Deletion of this sequence abolishes glucocorticoid induction of transcription^{4–8}. According to a current model, activation of the cytoplasmic receptor protein by hormone binding leads to its increased affinity for and translocation to the nucleus⁴. However, recent reports that the oestradiol and progesterone receptors are localized in the nucleus in the absence of steroid^{9–11} led us to examine whether the free receptor interacts *in vivo* with its DNA binding site in the absence of hormone binding. We used the genomic footprinting technique^{12–15} to show that changes in *in vivo* protein–DNA interactions within the GREs of the tyrosine aminotransferase gene (*TAT*) can be detected only after hormone treatment in hepatoma cells. Such changes are not detected in fibroblast cells, in which the *TAT* gene is not expressed. Many of the changes in dimethylsulphate reactivity observed in the living cell are also found *in vitro* using cloned DNA and a partially purified glucocorticoid receptor.

The GREs of the rat *TAT* gene are located unusually far upstream, at –2.5 kilobases (kb), from the transcriptional start site (Fig. 1). These elements were initially indicated by the appearance of a DNaseI-hypersensitive site following hormone treatment¹⁶ and then identified by gene transfer experiments and DNaseI footprinting using partially purified glucocorticoid receptor complex *in vitro*¹⁷. Sequence analysis revealed several sequence motifs that fit the established GRE consensus sequence^{1,2,4}. In this study we focus on those elements (II and III, Fig. 1) that have been shown to be essential for glucocorticoid induction by gene transfer experiments.

The instability during purification of the glucocorticoid receptor in the absence of ligand has hindered the analysis of its potential interaction with DNA sequences by *in vitro* methods, but the recent development of the genomic footprinting technique^{12,15} has made possible the analysis of receptor–DNA interactions *in vivo* in the absence of steroid. Cells of the *TAT*-expressing hepatoma cell line FTO-2B were withdrawn from glucocorticoids by incubation for at least 16 hours in serum-free medium before induction with the steroid dexamethasone for various times. Trypsinized cells suspended in medium were reacted with dimethyl sulphate (DMS) to probe for the reactivity of the N7 position of guanines in the major groove of the DNA helix^{15,19,20}. Patterns of guanine (G) residues were obtained using the probe fragment indicated in Fig. 1.

Figure 2a shows an example of such an analysis of uninduced and induced FTO cells. The band intensity reflects the reactivity of a given guanine or a group of unresolved guanines for the chemical modification. Protection from methylation at guanines is most readily explained by protein binding whereas enhanced modification of guanines has been interpreted as a local increase in reagent concentration in a hydrophobic pocket caused by close contacts of protein to DNA²⁰. Methylation protection and enhancement after hormone treatment were analysed quantitatively for each guanine (Fig. 2c). The data were collected from four independent experiments, each DNA sample being analysed on at least two blots. A number of changes in band intensity

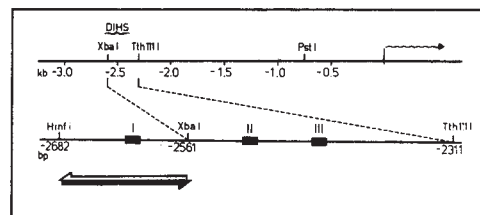


Fig. 1 The 5'-flanking region of the rat *TAT* gene. Upper line, 3 kb of the 5'-flanking region of the rat *TAT* gene: Numbering, distance from the cap-site; wavy arrow, the start of transcription; DIHS, region of dexamethasone-induced DNaseI hypersensitivity¹⁶. The lower line, enlargement of the region around the dexamethasone-inducible DNaseI-hypersensitive site: black boxes, sequence homologies to the GRE consensus sequence labelled with roman numerals; arrows, single-stranded DNA probes used to generate genomic sequence synthesized from an M13 vector containing the *HinfI*–*XbaI* fragment.

appear to be correlated with hormone treatment. DMS protections are evident at the sites of glucocorticoid interaction as identified by DNaseI footprinting¹⁷. Additional changes in reactivity map to neighbouring sequences (see Fig. 4). In a non-expressing rat fibroblast cell-line (XC cells) no such changes could be discerned after hormone treatment (Fig. 2a), nor were they observed when FTO-2B cells were treated with the glucocorticoid antagonist RU486 (Fig. 2b).

To ascertain whether the changes in DMS reactivity observed *in vivo* at the *TAT* gene GREs are caused by the glucocorticoid receptor, we performed *in vitro* methylation experiments, using cloned *TAT* DNA fragments containing the GREs and a partially purified glucocorticoid receptor (Fig. 3). The most prominent G protections map to the conserved TGTTCT motif and are identical with those shown to be contacted by the receptor in the GRE of mouse mammary tumour virus (MMTV)²¹.

Figure 4 summarizes the data obtained *in vivo* and *in vitro*. Comparison of *in vivo* G reactivity patterns from uninduced and induced FTO-2B cells clearly shows that protection of guanines in the binding site is seen after dexamethasone treatment of cells (Fig. 2). They are accompanied by a number of hormone-induced changes in reactivity in neighbouring sequences. The most dramatically enhanced G reactivities are seen at four adjacent guanines (–2,459 to –2,456) between the two receptor binding sites. Most of the changes in DMS reactivity following hormone treatment are also found *in vitro* using a partially purified glucocorticoid receptor. The changes are not found in XC cells, where the *TAT* gene is not transcribed. These findings strongly indicate that changes in reactivity at guanines within the GRE are indeed caused by interaction of the glucocorticoid receptor with its binding site and that its affinity in the absence of hormone binding is too low to lead to a tight interaction with a GRE at receptor concentrations found in the living cell.

Receptor binding is accompanied by changes in chromatin structure that render a region of 200 base pairs (bp) surrounding the GREs hypersensitive to DNaseI digestion¹⁶. Whether this altered structure is a prerequisite for receptor binding or rather a consequence of binding is not known. For the MMTV GREs a similar DNaseI hypersensitivity has been shown to precede or parallel hormonal stimulation of MMTV promoter function¹⁸.

It should be emphasized that these effects on guanine reactivities have been observed within a region which is unusually remote (at –2.5 kb) from the cap-site that is selectively rendered hypersensitive to DNase I digestion after dexamethasone treatment. Using the same DNA samples no hormone-dependent DMS effects were seen within several hundred nucleotides surrounding the *TAT* gene cap-site or within a region of DNaseI hypersensitivity at –1,000 bp (data not shown). Within the first

Fig. 2 Glucocorticoid-induced changes in DMS reactivity *in vivo*. **a**, G ladders for both strands of a region of genomic DNA between -2,380 and -2,540 bp (upstream) of the transcriptional start site of the *TAT* gene from DMS-treated FTO-2B or XC cells (+, induced with dexamethasone; -, uninduced); N, *in vitro* methylated protein-free DNA. Protection (Δ) and enhancement ($\blacktriangle$) seen when comparing dexamethasone-induced with uninduced FTO-2B cells; numbers, distance from the cap-site. Only those reactivity changes that appeared highly significant in the quantitative analysis (c) are indicated. *, Band originating from hybridization to *lacZ* sequences on a contaminating fragment; hatched bars, positions of the two major DNaseI footprints obtained *in vitro* with partially purified glucocorticoid receptor¹⁷. **b**, As in **a**, but only FTO-2B cells were used and incubation with the glucocorticoid antagonist RU486 (RU) was carried out in parallel with dexamethasone inductions. **c**, Quantitative analysis of hormone-dependent changes in G methylation; summary of results. Nucleotide numbers (from -2,560 to -2,390) upstream of the *TAT* cap-site are given on the abscissa. Hatched bars, sites of receptor binding identified by DNaseI footprinting *in vitro*¹⁷; black bar, conserved hexanucleotide TGTCT. For each G residue the logarithm between the median of induced divided by the median of uninduced samples ($\log +\text{dex}/-\text{dex}$) is plotted. Δ and ∇ symbolize this value for each guanine on the upper and lower strand, respectively. Positive values indicate dexamethasone-dependent enhancements, negative values represent inducible protections. The significance of the deviations obtained was checked by comparing induced and uninduced values using a Kruskal-Wallis test²⁴. Those values that fell below a threshold of 5% probability of error (1st order error) are indicated with vertical lines.

Methods. FTO-2B and XC cells were grown to about 80% confluency in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf-serum and 10 mM HEPES pH 7.4, withdrawn in serum-free DMEM for at least 16 h and incubated for 4 h with serum-free DMEM with or without 10^{-6} M dexamethasone or 10^{-6} M RU486. Induction times were 20 min–12 h, no significant time-dependent changes in the DMS reactivity pattern were observed (data not shown). DMS treatment of whole cells and *in vitro* methylation of protein-free DNA were as published^{15,19}. After isolation of cellular DNA, 25 μ g of each sample was restricted with *HinfI* (Boehringer), reacted with piperidine and separated on a sequencing gel¹². Details of DNA blotting onto Gene Screen membrane (NEN) UV crosslinking, hybridization and washing of filters were as in ref. 12. Probe synthesis from a single-stranded M13 template was performed as described¹² with modifications: 100 mM NaCl was included in primer annealing and elongation reactions, 250 μ Ci of [α -³²P]dATP (NEN), 5,000 Ci mmole⁻¹ was used per probe synthesis. The newly-synthesized probe DNA was separated from the template strand in a 6% denaturing gel and recovered from the polyacrylamide by isotachopheresis²². Exposure times were 7–14 days with 'lightning plus' intensifying screens (DuPont). Quantitative analysis was done as Giniger *et al.*²³; four independent DMS treatments of FTO-2B cells were performed under either dexamethasone-induced or uninduced conditions. The resulting DNAs were analysed on at least two independent blots. Genomic sequencing ladders were scanned with an Elscript 400 densitometer (Fisher Scientific) at 16 μ m resolution. Peak integrals were calculated and median values determined for each guanine band.

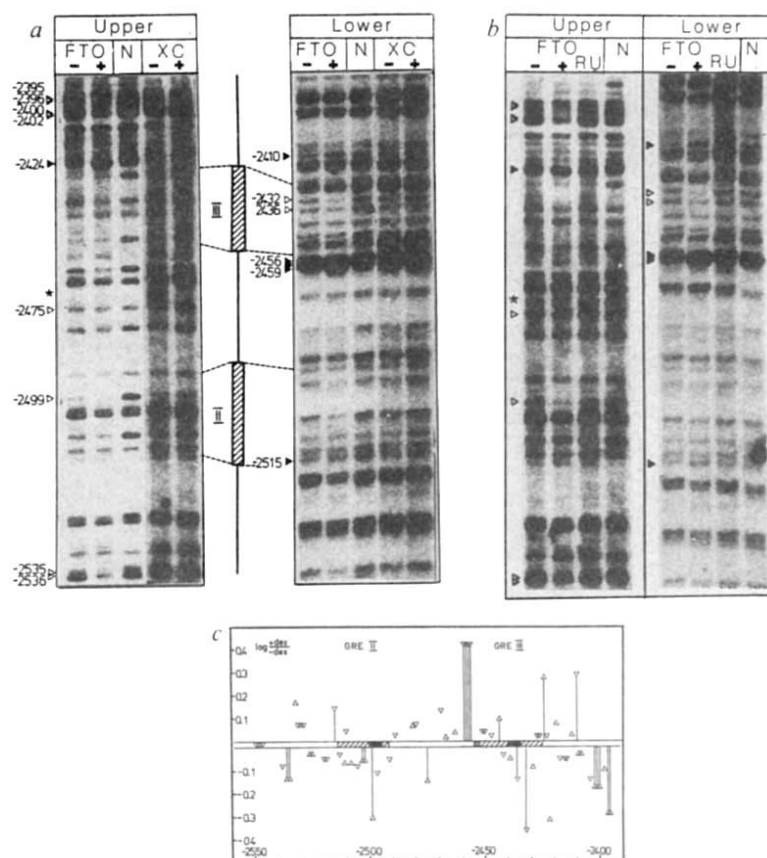
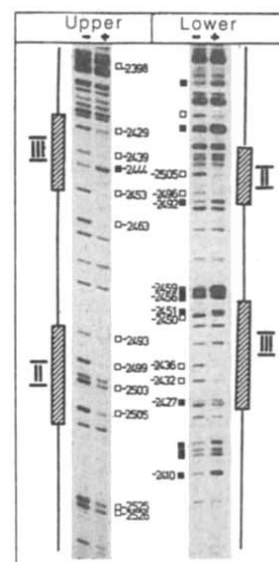


Fig. 3 Changes in DMS reactivity following glucocorticoid receptor binding *in vitro*. Left panel, results obtained for the upper strand and right panel, for the lower strand with (+) and without (-) triamcinolone-saturated glucocorticoid receptor. Protected guanines are indicated by open squares, enhanced methylation at a guanine as a filled square. Guanines that are influenced in their reactivity when comparing the '-' and '+' lanes are labelled with their numbers upstream of the *TAT* cap-site. Hatched bars, regions protected against DNaseI-digestion by bound glucocorticoid receptor *in vitro*¹⁷. **Methods.** Purification of the glucocorticoid receptor²⁵ included as a final step DEAE-Sepharose 6 CL column chromatography in which the receptor eluted as a single peak at 150 mM NaCl. A *XbaI*-*Tth111I* fragment containing both functional GREs was isolated from a subclone and end-labelled using T4 kinase polynucleotide and [γ -³²P]ATP either at the *XbaI* (upper strand) or at the *Tth111I* (lower strand) sites. Labelled fragment (0.5–1.0 fmol) was incubated with (+) or without (-) 90 fmoles of triamcinolone-saturated receptor in a volume of 25 μ l containing 100 mM NaCl, 0.6 mM EDTA, 7% glycerol (w/v), 22 mM Tris-Cl, 1 mM MgCl₂, 0.5 μ g bovine serum albumin 6 mM β -mercaptoethanol, 0.1 mM dithiothreitol, 1 μ M triamcinolone, 15% polyethyleneglycol 6000 (Serva), pH 8.2. After 45 min at room temperature, 2 μ l of 10% DMS (Fluka) was added and reaction stopped after 2 min incubation at room temperature by adding 75 μ l buffer containing 400 mM sodium acetate, 140 mM β -mercaptoethanol and 270 μ g ml⁻¹ transfer RNA. DNA was extracted with phenol/chloroform (1:1), precipitated and subjected to piperidine cleavage according to Maxam and Gilbert¹⁹, and finally dissolved in formamide containing loading buffer and analysed on a 6% sequencing gel.



200 bp upstream of the transcriptional start site several protections and enhancements can be identified that are strictly correlated with the active state of the gene in FTO-2B cells but are not seen in XC cells. However, none of them are influenced by hormone treatment (P.B., unpublished data).

Comparison of the methylation patterns of the two analysed GREs *in vivo* reveals qualitative and quantitative differences.

Whereas the protection of the central guanine (-2,499) in element II is clearly evident, it is less prominent in element III (-2,436). A surprising finding is a very strong protection of a TGT (-2,432) upstream of the conserved hexanucleotide TGTCT of GRE III. Why the two binding sites behave so differently is not known. Recent results suggest that this difference in DMS reactivities might reflect differences in the

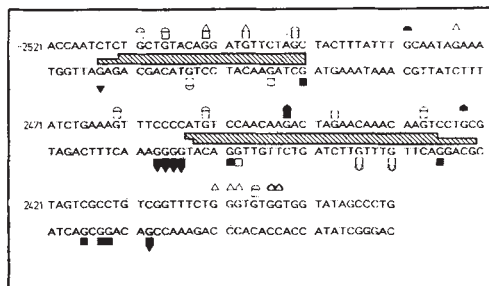


Fig. 4 Summary of results obtained by DMS reactivity experiments *in vivo* and *in vitro*. Changes in DMS reactivity at guanines in the DNA sequence between nucleotides -2,521 and -2,382, upstream of the *TAT* cap-site, are shown. Hatched bars, sites of glucocorticoid receptor interaction as identified by *in vitro* footprinting experiments¹⁷. Filled symbols, G residues showing enhanced methylation; open symbols, guanines showing protection from methylation. Squares, *in vitro* data; triangles, dexamethasone-dependent changes *in vivo*; half-circles, changes in reactivity found *in vivo* comparing the pattern obtained from uninduced cells with that from protein-free DNA. For the *in vivo* data only those guanines are indicated that by quantitative analysis have been shown to be significantly influenced in their reactivity (Fig. 2c).

inducing capacity of the two GREs (M. Jantzen and U.S., unpublished data).

In addition to effects in the receptor binding site, a number of changes in reactivity are observed in neighbouring sequences after dexamethasone treatment. Most prominent among these are the enhancements of guanines at -2,515, -2,459 to -2,456, and at -2,424 and protections of guanines at -2,475, -2,402 to -2,400, -2,396, and -2,395, *in vivo* and to some extent *in vitro*. Whether these changes are due to alterations in DNA structure as a consequence of glucocorticoid receptor binding or interaction with other factors not yet defined remains unclear.

These changes in DMS reactivity are clearly correlated with hormone induction and other changes are evident when the pattern of DMS reactivity of uninduced FTO-2B cell chromatin is compared with that of protein-free FTO-2B DNA (quantitative analysis not shown). Analysis of the reactivity of single guanines in naked genomic DNA at various sites and in plasmid DNA revealed that guanines flanked by thymidines or followed by a thymidine are often unusually sensitive to methylation. This enhanced reactivity is not observed *in vivo*, which might be explained by the difference in reaction conditions inside living cells compared with protein-free DNA *in vitro*. Because of these differences in methylation conditions naked genomic DNA cannot serve as a suitable standard for comparisons of DMS reactivity obtained *in vivo*. The methylation pattern in uninduced FTO-cells is identical to that obtained from induced or uninduced XC-cells. In contrast to observations at the cap-site, there is no evidence for an interaction of protein factors with the receptor binding site in the absence of hormone that discriminates *TAT* in FTO-cells from the gene in nonexpressing fibroblast cells.

We have applied genomic sequencing methodology to obtain information on the *in vivo* interaction of the glucocorticoid receptor with its recognition sequence in the *TAT* gene. Results support the concept that glucocorticoid increases the affinity of the receptor for its target sequence. These experiments do not exclude the possibility that the unliganded receptor can interact with a GRE, albeit with lower affinity and selectivity.

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Steroid-free glucocorticoid receptor binds specifically to mouse mammary tumour virus DNA

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Steroid hormones are thought to modulate gene expression through their interaction with receptor proteins. The intracellular localization of unoccupied receptor proteins has been a subject of controversy: free glucocorticoid receptor appears to reside in the cytoplasm and moves to the cell nucleus only after binding the steroid^{1,2}. The purified hormone-bound glucocorticoid receptor has been shown to bind selectively to hormone regulatory elements (HRE) in the vicinity of hormonally-inducible promoters and, in particular, in the long terminal repeat (LTR) region of mouse mammary tumour virus (MMTV)^{3,4}. We have tackled the question of whether the hormone itself is required for the interaction of the receptor protein with the HRE. Using monoclonal antibodies to the receptor we find that upon heat-activation the steroid-free glucocorticoid receptor present in rat liver cytosol binds specifically *in vitro* to the HRE of MMTV. No qualitative differences in the DNaseI-footprints were detected when hormone-free receptor was compared to the hormone-receptor complex or even receptor complexed with the hormone antagonist RU486. We conclude that the steroid ligand is not an absolute requirement for generating the conformation of the glucocorticoid receptor that allows its interaction with the HRE *in vitro*. An alternative function of the hormone *in vivo* could be to modulate nuclear partitioning of the receptor.

To determine whether the steroid ligand is required for specific binding of the receptor to DNA it is important to use receptor that has been subjected to as few manipulations as possible, because the steps required for receptor purification could induce artificial conformational changes of the receptor protein, and thus alter its behaviour in DNA-binding studies. Therefore, we decided to use the receptor in crude hepatic cytosol from adrenalectomized rats. To show that the glucocorticoid receptor in this cytosol is free of ligand we used a competition assay. Cytosol was heated at 100 °C for 4 min to denature the steroid-binding proteins and then extracted with *n*-butanol. The amount of ligand with affinity for the glucocorticoid receptor in the

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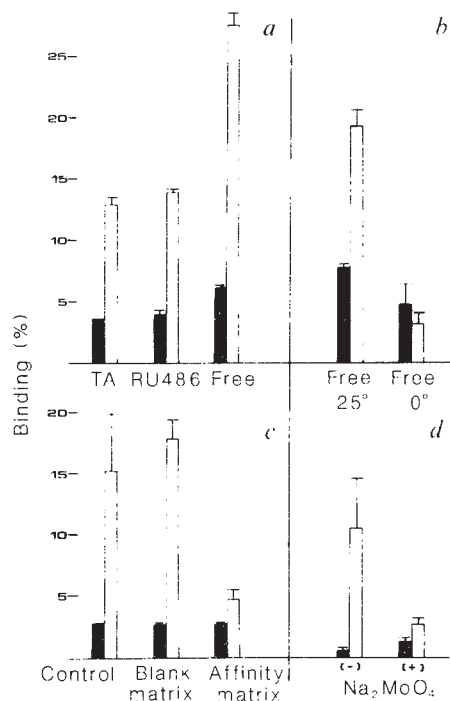


Fig. 1 Immunological detection of DNA-receptor complexes. *a*, DNA-cellulose filtered hepatic cytosol from adrenalectomized rats, either charged with 50 nM [³H]triamcinolone acetonide (TA) or 100 nM RU468 (RU468) or free of hormones (FREE), was activated at 25 °C and aliquots incubated with radioactive DNA fragments derived from plasmid p13-13⁶, which contains parts of the *env* gene and the LTR region of MMTV. Monoclonal antireceptor antibodies were added and after incubation precipitated with a second antibody linked to Pansorbin. Bound radioactivity was counted. The average and variation in duplicate measurements are shown. Black bars, binding of a 2.7 kb control fragment (vector sequences and part of the *env* gene), not selectively bound by purified glucocorticoid receptor⁴. Open bars, binding of a 2.3 kb fragment containing the LTR region of MMTV together with mouse genomic sequences and the herpes simplex virus (HSV) *tk* gene. *b*, Left, as in *a* with hormone-free cytosol (25 °C); right, the same but performed at 0 °C during all the incubations to prevent thermal activation (0 °C). *c*, Experiment with uncharged cytosol preincubated at 4 °C for 16 h in presence of 15 vol% of pure Sepharose 4B (Blank matrix), Sepharose 4B containing 0.5 mg ml⁻¹ of immobilized dexamethasone (Affinity matrix) or in absence of Sepharose (Control). Shorter DNA fragments were used: black bars, 450 base pairs (bp), *env* and vector sequences; open bars, 600 bp, MMTV-LTR and mouse genomic sequences. *d*, Hormone-free cytosol activated and incubated in the absence (-) or the presence (+) of 20 mM NaMoO₄ and with DNA fragments as in *c*. **Methods.** Cytosol was passed through DNA-cellulose and receptor content determined by the charcoal technique⁵. Aliquots containing 10 ng of receptor with or without steroid ligand were heat activated at 25 °C for 30 min and incubated for additional 30 min with 10 ng of the end-labelled restriction fragment and 30 ng (with the large restriction fragments, Fig. 1*a, b*) or 1 µg (with the short fragments, Fig. 1*c, d*) of calf thymus DNA (sheared to an average size of 0.5 kb kilobase (kb) by treatment with 0.3 M NaOH at 100 °C for 30 min) in a final volume of 50 µl containing: 10 mM Tris HCl pH 7.5, 0.1 mM Na₂ EDTA & 110 mM NaCl. After the addition of 50 ng of Protein-A-Sepharose purified monoclonal antireceptor antibody IGR49/4 the incubation was continued at 25 °C for 15 min. The assays were rotated at 4 °C with 1 µl of solid Pansorbin containing 50 µg of purified rabbit anti mouse IgG in a final volume of 200 µl containing: 12 mM HEPES, pH 6.8, 150 mM NaCl and at 0.5–1 µg calf thymus DNA. After centrifugation the pellets were washed with Tris-buffer containing 1 mg ml⁻¹ bovine serum albumin and 110 mM NaCl.

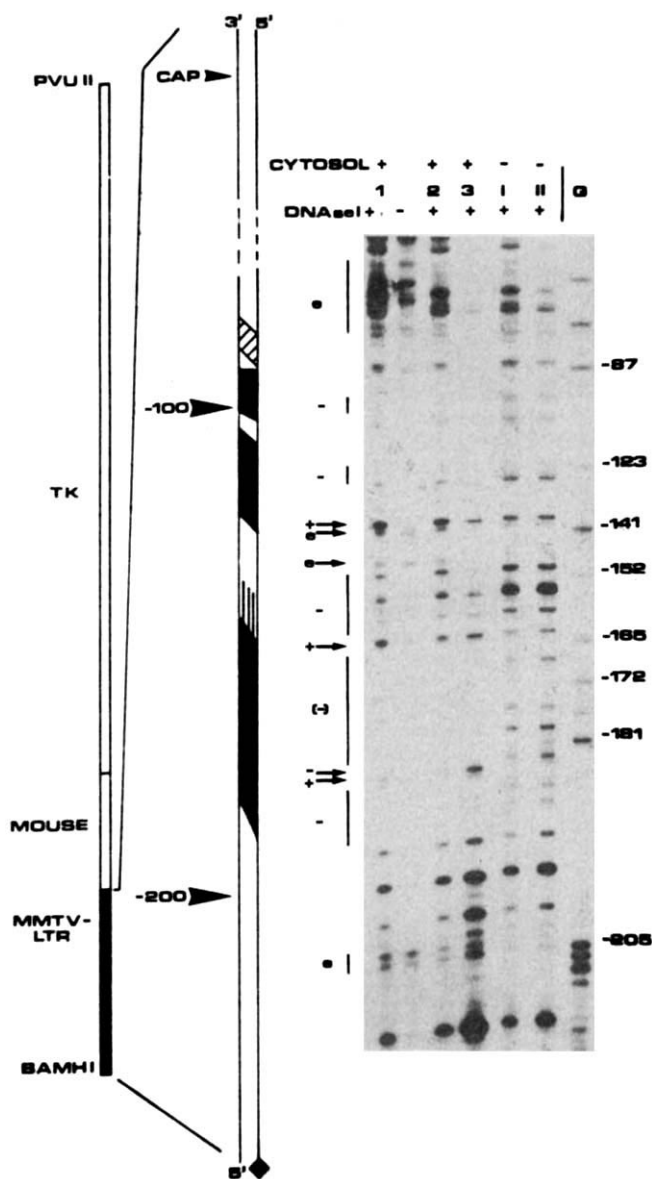


Fig. 2 DNaseI footprints with crude cytosol receptors. DNaseI footprints generated by the receptor complexed with corticosterone (lane 1+), with RU468 (lane 3) or free of hormone (lane 2). Controls: Lane 1-, like lane 1+ but without added DNaseI; lanes I, and II, DNaseI digestions in absence of cytosol. Lane G: guanosine-specific sequencing reaction¹⁹. The fragment used and position of the label (♦) are shown at the left. -, regions protected by the receptor; +, hypersensitive ones; e, cuts by endogenous nucleases (lane 1-). Numbers refer to the distance from the initiation of transcription (CAP). **Methods.** The 2.3 kb fragment of p13-13 was 3'-labelled using dGTP, [³²P]dATP and the Klenow fragment of DNA-polymerase I. It was used in an assay like that in Fig. 1*a*. The precipitate was resuspended in 80 µl of washing buffer and calf thymus (1 µg) and MgCl₂ (final concentration 2.3 mM) in 20 µl were added. After the addition of 0.1 µg DNaseI (Worthington) and incubation at 20 °C for 60 sec the digestion was stopped with 3 µl 250 mM Na₂ EDTA. The DNA was solubilized with 0.6% SDS and 0.3 M NaCl and purified by cooling to 0 °C, centrifugation, fivefold extraction with chloroform/isoamylalcohol and precipitation. After digestion with Pronase P the samples were analysed in a 6.5% polyacrylamide-urea sequencing gel¹⁹. For lanes I and II 30 ng of labelled DNA were digested in an assay volume of 300 µl with 0.4 µg (I) or 0.9 µg (II) DNaseI.

Table 1 Glucocorticoid receptor ligand in cytosol from adrenalectomized rats

Competitor added	Corticosterone	<i>n</i> -Butanol extract	Binding of [³ H] triamcinolone acetoneide	
			c.p.m.	%
—	—	—	3,215 (154)	100
1 ng	—	—	1,954 (2)	61
2 ng	—	—	1,418 (74)	44
5 ng	—	—	865 (12)	27
25 ng	—	—	412 (8)	13
—	—	2 ml	3,468 (247)	108
—	—	2 ml*	3,140	98

Aliquots of rat liver cytosol from adrenalectomized rats were incubated with [³H]triamcinolone acetoneide in the presence of corticosterone or *n*-butanol extract from 2 ml of cytosol. At equilibrium the protein bound radioactivity was determined by the charcoal technique. Values are average and variation of duplicate determinations⁵. To obtain cytosol, male Wistar rats (body weight 180–200 g) were adrenalectomized, maintained under standard conditions with physiological saline *ad libitum* and killed after four days. The livers were perfused through the portal vein with 20 ml cold homogenization buffer (20 mM sodium phosphate, pH 7.0, 1 mM Na₂EDTA, 50 mM NaCl, 2 mM β -mercaptoethanol, 2 mM dithioerythritol and 10% glycerol), removed, minced and homogenized in three volumes of the same buffer. After centrifugation at 109,000 g and 4 °C for 90 min, the supernatant was passed through 0.14 volumes of calf-thymus-DNA-cellulose (0.5 mg DNA per g cellulose). The flow-through was collected and stored in liquid nitrogen. For *butanol extracts*, 2 ml aliquots of cytosol were heated at 100 °C for 4 min, extracted with one volume *n*-butanol by vortexing for 30 s and concentrated by evaporation. A control extraction with charcoal-treated cytosol supplemented with [³H]cortisol (200 ng, 0 °C for 60 min) showed that this procedure extracts 86% of the hormone. For the competition assay, cytosol was treated with 10% dextran-coated charcoal⁵ at 0 °C for 5 min, and aliquots (100 μ l) were preincubated for 5 min in the presence of either corticosterone or *n*-butanol extract, before addition of 0.9 ng of [³H]triamcinolone acetoneide (Amersham Büchler, specific activity 1.1 TBq mmol⁻¹) and further incubation for 60 min at 0–4 °C. The amount of protein-bound radioactivity was determined by the charcoal procedure⁵.

* In this experiment the *n*-butanol extract was added only 5 min prior to the charcoal treatment to exclude an effect of the organic extract *per se* on the efficiency of the test.

organic extract was determined by measuring its ability to compete with ³H triamcinolone acetoneide for binding to liver cytosol treated with charcoal⁵. In the hepatic cytosol we used there is less than 0.5 ng ml⁻¹ of ligand, so that given the association constant of the receptor for corticosterone, at least 98% of the receptor molecules are free of steroid (Table 1). The unoccupied receptor molecules are able to bind steroid, as shown by their retention in a steroid affinity matrix (Fig. 1c, below).

For DNA-binding experiments we used two end-labelled restriction fragments of approximately equal size derived from a plasmid carrying the LTR region of MMTV (Fig. 1, ref. 6). The individual fragments were incubated with hormone-free cytosol or with cytosol that was precharged with either the synthetic glucocorticoid triamcinolone acetoneide or the potent antagonist RU486⁷. In every case the cytosol was incubated at 25 °C for 30 min before the addition of DNA. The complexes of receptor and DNA were immunoprecipitated with mouse monoclonal antibodies against the receptor, IGR49/4⁸, and rabbit antimouse immunoglobulin linked to Pansorbin (Calbiochem). The radioactivity in the precipitates was measured and is expressed as percentage of the total radioactive DNA. In all experiments the restriction fragment containing LTR sequences was immunoprecipitated three to four times more efficiently than the slightly larger vector fragment (Fig. 1a). The hormone-free receptor binds specifically to the LTR-fragment, and exhibits a total DNA-binding activity higher than that of

the cytosol incubated with either receptor ligand. This result is not influenced by pre-treatment of the cytosol with dextran-coated charcoal (data not shown), nor by using shorter restriction fragments that increase the specificity of DNA binding (Fig. 1c, d). No significant differences were found between the DNA binding properties of cytosol charged with agonist or antagonist ligands (Fig. 1a).

Specific binding to the LTR fragment was dependent upon previous heat activation of the hormone-free cytosol and could be prevented by addition of 20 mM sodium molybdate (Fig. 1b, d). Binding of the antibody *per se* does not activate the receptor by artificial conformational change (Fig. 1b). That the receptor molecules responsible for specific DNA binding in hormone-free cytosol are able to bind steroid was shown by passing the cytosol through an affinity matrix of immobilized dexamethasone⁹. This treatment removes from the cytosol 85% of the steroid binding activity, eliminates any immunoreactive material in Western blots (data not shown), and dramatically decreases specific DNA binding (Fig. 1c). Thus, a steroid-free form of the glucocorticoid receptor is able to bind active hormone and interacts selectively with the LTR of MMTV. These results were confirmed by a nitrocellulose filter binding assay which did not depend on the use of antibodies (data not shown).

To analyse in more detail the interaction of the different receptor forms with the LTR region we performed DNase I footprinting experiments with the immunisolated protein-DNA complexes. Though the quality of the footprints is not comparable to those obtained with purified receptor, the limits of the region protected against DNase I are the same as those previously reported⁴, and do not differ in the footprints obtained with hormone-free cytosol or with liganded cytosols (Fig. 2). Small quantitative differences between the protection in the presence of agonist and antagonist ligands can not be excluded by these results.

We conclude that the hormone-free glucocorticoid receptor in crude rat liver cytosol binds *in vitro* to the same regulatory elements of the MMTV-LTR region that have been shown to mediate hormonal regulation of transcription *in vivo*^{3,4,6}. Our results apparently contradict previous reports, based on binding of radioactive steroid-receptor complexes to an excess of calf thymus DNA-cellulose^{10,11}, that binding of the hormone to the receptor is a prerequisite for the complex to be 'activated' and bind to DNA. We have repeated these experiments and find that the steroid-free receptor can be heat activated and bind to DNA-cellulose, though the extent of binding is only 20–40% of that observed with the hormone-receptor complexes (data not shown). One possible reason for the quantitative discrepancy with the results presented here could be that an excess of calf thymus DNA-cellulose acts as an ionic exchanger rather than a specific ligand for the DNA-binding domain of the receptor. This could also explain the difference between our results and a previous report claiming that, in a DNA-cellulose competition assay, the receptor complexed to the antagonist RU486 binds less efficiently to MMTV-LTR than receptor complexed to agonist¹².

Our results confirm the requirement for an *in vitro* heat activation step in order for the receptor, whether free or steroid-bound, to bind to the DNA regulatory sequences. At physiological body temperatures receptor activation *in vivo* should be spontaneous. In the absence of hormone the receptor seems to be localized in the cytoplasm, so that some mechanism preventing receptor activity must exist *in vivo*.

It is conceivable that in the absence of hormone the receptor is complexed to other cellular factors or structures that prevent its interaction with the physiological nuclear targets. Candidates for this role are RNA and the 90 kilodalton heat shock protein, which have been found associated with the non-activated form of several steroid receptors^{13,14}. The function of the hormone could be to dissociate the receptor from the 'anchoring' structure, and thus to allow its translocation to the cell nucleus. As a novel

variation of this concept, the actual function of the hormone may be to induce a conformational change of the receptor that exposes a nuclear translocation signal. Amino acid sequences responsible for nuclear translocation have been identified in several nuclear proteins, and appear to be distinct from the actual DNA-binding site^{15,16}. In the absence of the hormone this hypothetical nuclear translocation signal would be masked and the receptor protein would be excluded from the cell nucleus. The possible role of receptor phosphorylation in this remains an intriguing question.

Common to all of these models is the concept that the DNA binding domain of the receptor is functional in the absence of hormone. In the case of oestrogens there is evidence supporting a weak association of the steroid-free receptor with nuclear structures^{17,18}. Whether the hormone ligand is required for the activation of gene expression that follows DNA binding could be analysed in appropriate cell-free experiments.

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Molecular cloning and polymorphism of the human immune deficiency virus type 2

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We recently reported the isolation of a novel retrovirus, the human immune deficiency virus type 2 (HIV-2, previously named LAV-2), from patients with acquired immune deficiency syndrome (AIDS) originating from West Africa^{1,2}. This virus is related to HIV-1, the causative agent of the AIDS epidemic now spreading in Central and East Africa, as well as the USA and Europe (see ref. 3 for review) both by its morphology and by its tropism and *in vitro* cytopathic effect on CD4 (T4) positive cell lines and lymphocytes. But preliminary hybridization experiments indicated that there are substantiated differences between the sequences of the two genomes². Furthermore, the proteins of HIV-1 and HIV-2 have different sizes and their serological cross-reactivity is restricted to the major core protein, as the envelope glycoproteins of HIV-2 are not immunoprecipitated by HIV-1-positive sera^{1,2}. We now report the molecular cloning of the complete 9.5-kilobase (kb) genome of HIV-2, the observation of restriction site polymorphism between different isolates, and a preliminary analysis of the relationship of HIV-2 with other human and simian retroviruses.

A series of filter hybridizations of the HIV-2 RNA genome with probes derived from the complete cloned HIV-1 genome, as well as from regions expected to be well-conserved between HIV-1 and 2 (principally from the *gag* and *pol* genes) yielded extremely weak signals, even in conditions of very low stringency hybridization and washing². This made it very difficult to assess by Southern blot analysis the amount of HIV-2 viral and proviral DNA in infected cells. We therefore cloned a complementary DNA (cDNA) to the HIV-2 genomic RNA, to produce a specific hybridization probe. We used the strategy previously developed for the cloning of HIV-1 (ref. 4). An oligo(dT)-primed cDNA first strand was made in the detergent-activated endogenous reaction (using HIV-2 reverse transcriptase) with virions purified from supernatants of CEM cells (a lymphoblastoid CD4⁺ cell

line⁵) infected with the isolate ROD² and continuously producing high amounts of HIV-2. A collection of 10⁴ M13 recombinant phages was obtained and screened *in situ* with a labelled HIV-2 complementary DNA first strand. As previously done for HIV-1, we looked directly for HIV-2 specific clones, expected to represent an abundant group in the library, but detected only clones corresponding to cellular repetitive DNA. We can now explain this result by the low representation of HIV-2 specific clones in the library (less than 0.5%, compared to the 5-10% obtained in the cloning of HIV-1). Fortunately the high amounts of recombinant DNA produced by M13, its single-stranded nature, and the ability to perform extremely low stringency hybridization (at *T_m* -42 °C) on replica filters of M13 plaques allowed the successful use of an HIV-1 probe spanning 1.5 kb of the 3' end of the LAV_{BRU} isolate (Fig. 1a) to screen the library. About 50 positive plaques were detected, purified and characterized by end sequencing and cross-hybridizing of the inserts (Fig. 1). The different clones were found to be complementary to the 3' end of a polyadenylated RNA, having the AATAAA signal about 20 nucleotides upstream of the poly(A) tail, as found in the long terminal repeat (LTR) of HIV-1 (ref. 6). The part of the HIV-2 LTR that we sequenced is only very distantly related to the homologous domain in HIV-1 (Fig. 1b). Only ~50% of the nucleotides can be aligned, requiring the introduction of ~100 insertions and deletions. For comparison, the homology of the corresponding domains in HIV-1 isolates from USA and Africa is greater than 95%, with no insertions or deletions (M.A., unpublished data).

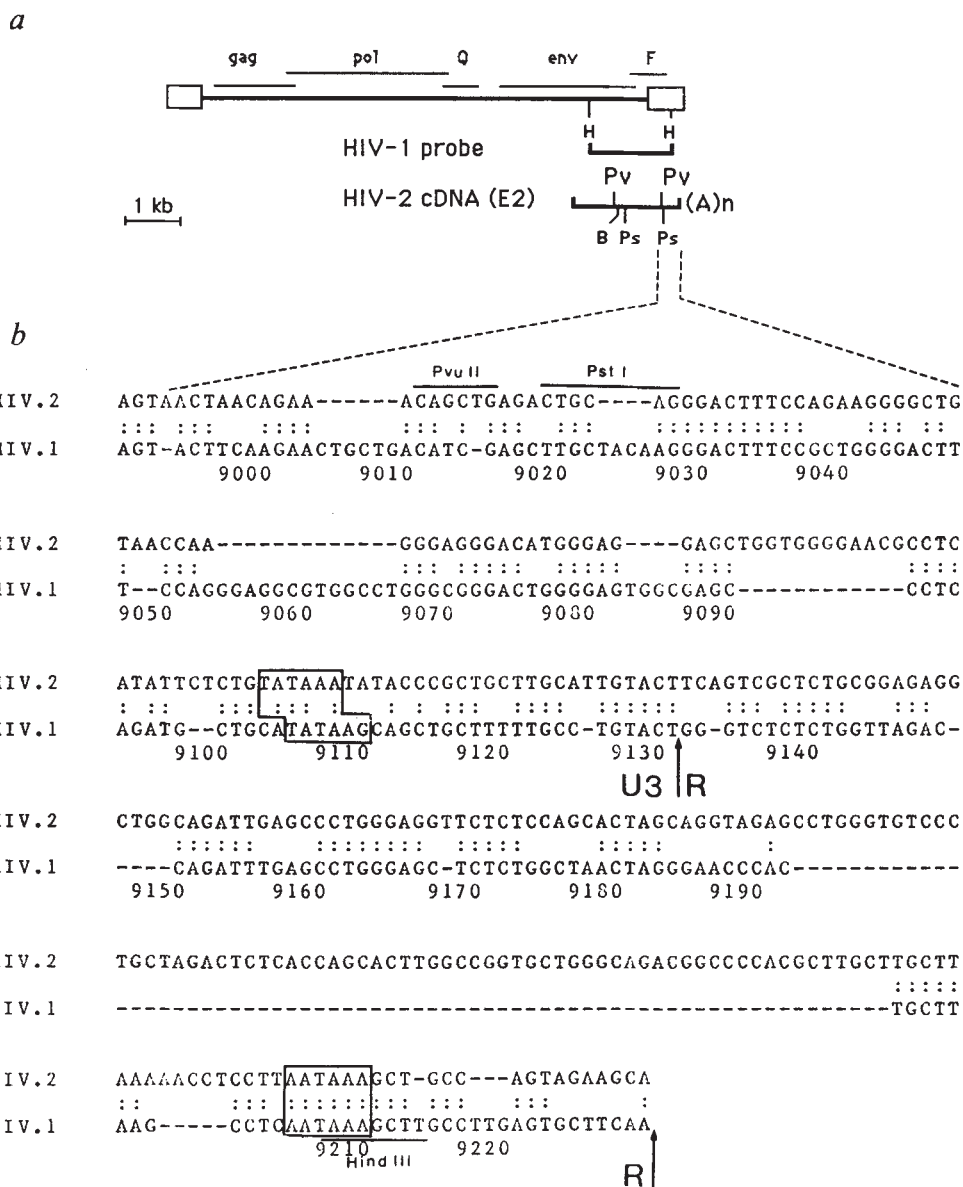
The largest insert of this group of M13 clones (E2, 2 kb) was used as a probe to demonstrate HIV-2 specificity in a series of filter hybridization experiments. First, this probe could detect the genomic RNA of HIV-2 but not HIV-1 in stringent conditions (Fig. 2C,D). Second, positive signals were detected in Southern blots of DNA from cells infected with the ROD isolate as well as other isolates of HIV-2 (Fig. 2A and Fig. 4A). No signal was detected with DNA from uninfected (not shown), or HIV-1 infected cells (Fig. 2A) confirming the exogenous nature of HIV-2. In undigested DNA from HIV-2 infected cells we detect principally a species of ~10 kb, probably corresponding to linear unintegrated viral DNA, and a species with an apparent size of 6 kb, probably the circular form of the viral DNA. Conversely, rehybridization of the same filter with an HIV-1 probe under stringent conditions showed hybridization to HIV-1 infected cells only (Fig. 2B).

To isolate the rest of the genome of HIV-2, a genomic library in lambda phage L47.1 (ref. 7) was constructed with a partial *Sau3AI* restriction digest of the DNA from the CEM cell line infected with HIV-2_{ROD}. About 2 × 10⁶ recombinant plaques

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Fig. 1 Cloned complementary DNA (cDNA) from the genomic RNA of HIV-2; **a**, genetic organization of HIV-1, position of the HIV-1 *Hind*III fragment used as a probe to screen the cDNA library, and restriction map of the HIV-2 cDNA clone, E2. B, *Bam*HI; H, *Hind*III; Ps, *Pst*I; Pv, *Pvu*II. **b**, partial nucleotide sequence of the LTR of HIV-2. The corresponding region of the HIV-1 LTR⁶ was aligned using the Wilbur and Lipman algorithm (window, 10; K-tuple, 7; gap penalty, 3; ref. 14). The U3-R junction in HIV-1 is indicated; poly(A) addition signal and potential TATA promoter regions are boxed.

Methods. HIV-2 virions were purified from 5 litres of supernatant from a culture of the CEM cell line infected with the ROD isolate² and a cDNA first strand using oligo(dT) primer was synthesized in a detergent-activated endogenous reaction on pelleted virus resuspended in 1 ml, as described⁴. RNA-cDNA hybrids were purified by phenol-chloroform extraction, and ethanol precipitation. The second-strand cDNA was done by the DNA polymerase I/RNAase H method¹⁵ and blunt-ended with T4 DNA polymerase using a commercial cDNA synthesis kit (Amersham). After attachment of *Eco*RI linkers (Pharmacia), *Eco*RI digestion, and ligation into *Eco*RI-digested dephosphorylated M13 tg130 vector (Amersham), a cDNA library was obtained by transformation of the *Escherichia coli* TG1 strain. Recombinant plaques (10⁴) were screened *in situ* on replica filters with the 1.5-kb *Hind*III fragment from clone J19, corresponding to the 3' part of the genome of the LAV_{BRU} isolate of HIV-1 (ref. 4), ³²P-labelled to a specific activity of 10⁹ c.p.m. µg⁻¹. The filters were prehybridized in 5× SSC, 5× Denhardt solution, 25% formamide, denatured salmon sperm DNA (100 µg ml⁻¹) at 37°C, for 4 h and hybridized for 16 h in the same buffer (T_m = 42°C) plus 4×10⁷ c.p.m. of the labelled probe (10⁶ c.p.m. per ml of hybridization buffer). The washing was done in 5× SSC, 0.1% SDS at 25°C for 2 h. 20× SSC is 3 M NaCl, 0.3 M Na citrate. Positive plaques were purified and single-stranded M13 DNA prepared and end-sequenced¹⁶.



were screened *in situ* with labelled insert from the E2 cDNA clone. Ten recombinant phages were detected and plaque-purified; three of them were characterized by restriction mapping and Southern blot hybridization with the E2 insert and probes from its 3' end (LTR) or 5' end (envelope), as well as with HIV-1 subgenomic probes (HIV-1 probes were used under non-stringent conditions). The λ ROD 4 clone is very likely to contain the complete genetic information of HIV-2, as it carries a 9.5-kb insert and is derived from a circular viral DNA. Two other clones, λ ROD 27 and λ ROD 35 are derived from integrated proviruses and carry a LTR and cellular flanking sequences and a portion of the viral coding sequences (Fig. 3A).

The relationship of HIV-2 to other human and simian retroviruses will be more precisely determined by the elucidation of the nucleotide sequence of their genomes, but some preliminary conclusions can already be drawn from hybridization experiments. The relative homology of the different regions of the HIV-1 and 2 genomes was determined by hybridization of fragments of the cloned HIV-1 genome with the labelled λ ROD 4

expected to contain the complete HIV-2 genome (Fig. 3B). Even at very low stringency (T_m = 42°C), the hybridization of HIV-1 and 2 is restricted to a fraction of their genomes, principally the *gag* gene (dots 1 and 2), the reverse transcriptase domain in *pol* (dot 3), the end of *pol* and the Q (or *sor*) genes (dot 5) and the F gene (or 3' ORF) and 3' LTR (dot 11). The HIV-1 fragment used to detect the HIV-2 cDNA clones contains the dot 11 subclone, which indeed hybridizes well to HIV-2 under non-stringent conditions. Only the signal from dot 5 persists after stringent washing. The envelope gene, but also the region of the *tat* gene and a part of *pol* seem very divergent. These data, and also the LTR sequence (Fig. 1B), indicate that HIV-2 is not an envelope variant of HIV-1.

We previously observed that HIV-2 is more related to the simian immune deficiency virus (SIV), than it is to HIV-1 (ref. 2). SIV (also designated simian T-cell lymphotropic virus type 3, STLV-3) is a retrovirus first isolated from captive macaques with an AIDS-like disease in the USA⁸. All the SIV proteins, including the envelope, are immunoprecipitated by sera from HIV-2

Fig. 2 HIV-2 specificity of the E2 cDNA clone. **A, B**, Southern blots of DNA extracted from CEM cells infected with isolates: HIV-2_{ROD} (a, c) HIV-2_{DUL} (b, d) and HIV-1_{BRU} (e, f); a, b, f, *Pst*I digested; c, d, e, undigested. **C, D**, dot blot hybridization of pelleted virions from CEM cells infected by the HIV-1_{BRU} (1), SIV isolate Mm 142-83 (3), HIV-2_{DUL} (4), HIV-2_{ROD} (5), HIV-1_{ELI} (6). Dot 2 is a pellet from an equivalent volume of supernatant from uninfected CEM. **A, C**, Hybridization with the HIV-2 cDNA (E2); **B, D**, hybridization to HIV-1 probe (*Sac*I 9-kb insert from HIV-1_{BRU}, ref. 4).

Methods. DNA was extracted from infected CEM cells continuously producing HIV-1 or 2, and 20 µg of *Pst*I-digested or undigested DNA, was electrophoresed on a 0.8% agarose gel, and transferred to nylon membrane. Virion dot blots were prepared in duplicate as described², by pelleting volumes of supernatant corresponding to the same amount of reverse transcriptase activity. Prehybridization was done in 50% formamide, 5×SSC, 5×Denhardt's solution, 100 µg ml⁻¹ denatured salmon sperm DNA for 4 h at 42 °C. Hybridization was performed in the same buffer plus 10% Dextran sulphate, and 10⁶ c.p.m. per ml of the labelled E2 insert (specific activity 10⁹ c.p.m. µg⁻¹) for 16 h at 42 °C. Washing was in 0.1×SSC, 0.1% SDS for 2×30 minutes. After exposition (16 h with intensifying screens), the Southern blot was dehybridized in 0.4 M NaOH, neutralized, and rehybridized in the same conditions to the HIV-1 probe labelled to 10⁹ c.p.m. µg⁻¹.

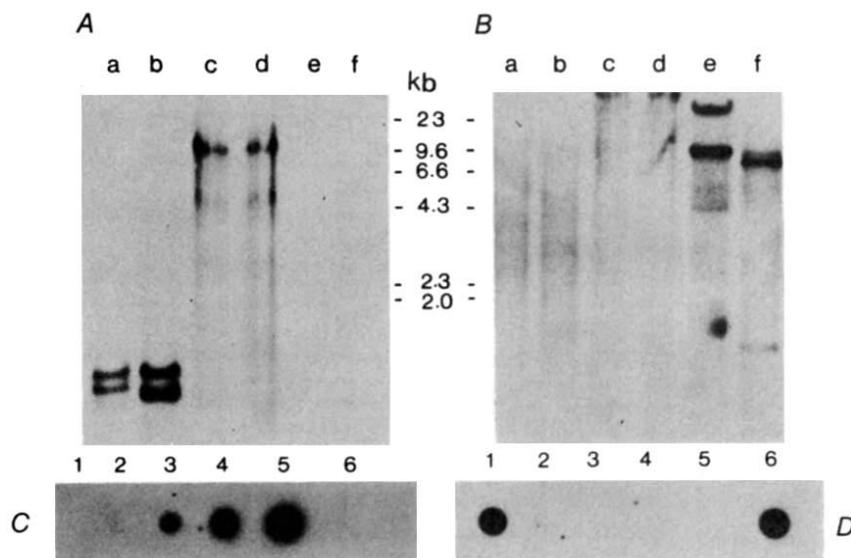
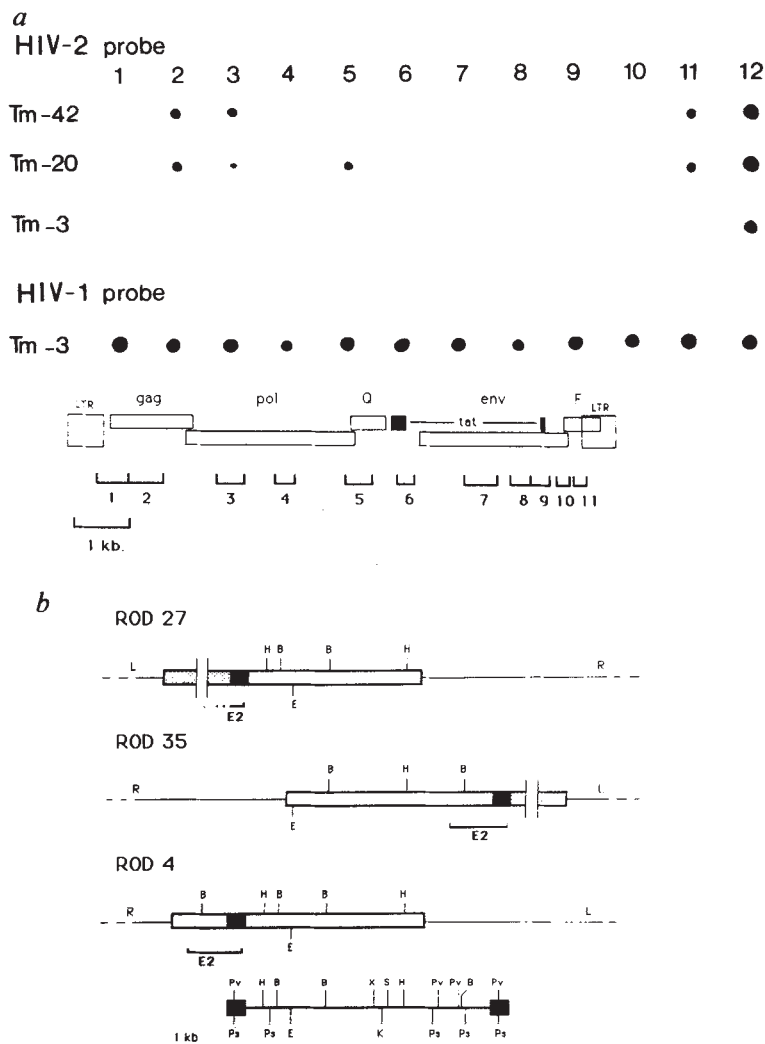


Fig. 3 Restriction map of the HIV-2_{ROD} genomes and similarity to HIV-1. **A**, Organization of three recombinant phage λ clones, ROD 4, ROD 27 and ROD 35; the open boxes represent viral sequence (the LTR are filled), whereas the dotted boxes represent cellular flanking sequences (not mapped); only some characteristic restriction enzymes sites are indicated. ROD 27 and 35 are derived from integrated proviruses whereas ROD 4 is derived from a circular viral DNA. The part of the λ clones that hybridizes to the cDNA E2 is indicated below the maps: a restriction map of the ROD isolate was reconstructed from those of the three λ clones. **B**, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; S, *Sac*I; X, *Xba*I. R, L, right and left *Bam*HI arms of the λL47.1 vector. **B**, 1-11: dots corresponding to the single-stranded DNA form of M13 subclones from the HIV-1_{BRU} cloned genome. Their size and position on the HIV-1 genome, determined by sequencing⁶, is shown below the figure. Dot 12 is a control containing λ phage DNA. The dot blot was hybridized in low stringency conditions (as described Fig. 1) with the complete λ ROD 4 clone as a probe, and successively washed in 2×SSC, 0.1% SDS at 25 °C. (*T*_m–42 °C), 1×SSC, 0.1% SDS at 60 °C. (*T*_m–20 °C), and 0.1×SSC, 0.1% SDS at 60 °C. (*T*_m–3 °C) and exposed overnight. A duplicate dot blot was hybridized and washed in stringent conditions (as described in Fig. 2) with the labelled λJ19 clone carrying the complete HIV-1_{BRU} genome⁴. HIV-1 and HIV-2 probes were labelled at the same specific activity (10⁸ c.p.m. µg⁻¹).

Methods. DNA from the HIV-2_{ROD}-infected CEM (Fig. 2, lanes a, c) was partially digested with *Sau*3AI; the 9–15-kb fraction was selected on a 5–40% sucrose gradient, and ligated to *Bam*HI arms of the λL47.1 vector⁷. Plaques (2×10⁶) obtained after *in vitro* packaging and plating on *Escherichia coli* LA 101 strain were screened *in situ* with the insert from the E2 cDNA clone. About 10 positive clones were plaque purified, and propagated on *E. coli* C600 recBC. The ROD 4, 27 and 35 clones were amplified and their DNA characterized by restriction mapping, and Southern blotting with the HIV-2 cDNA clone under stringent conditions, and *gag-pol* probes from HIV-1 used under non-stringent conditions.



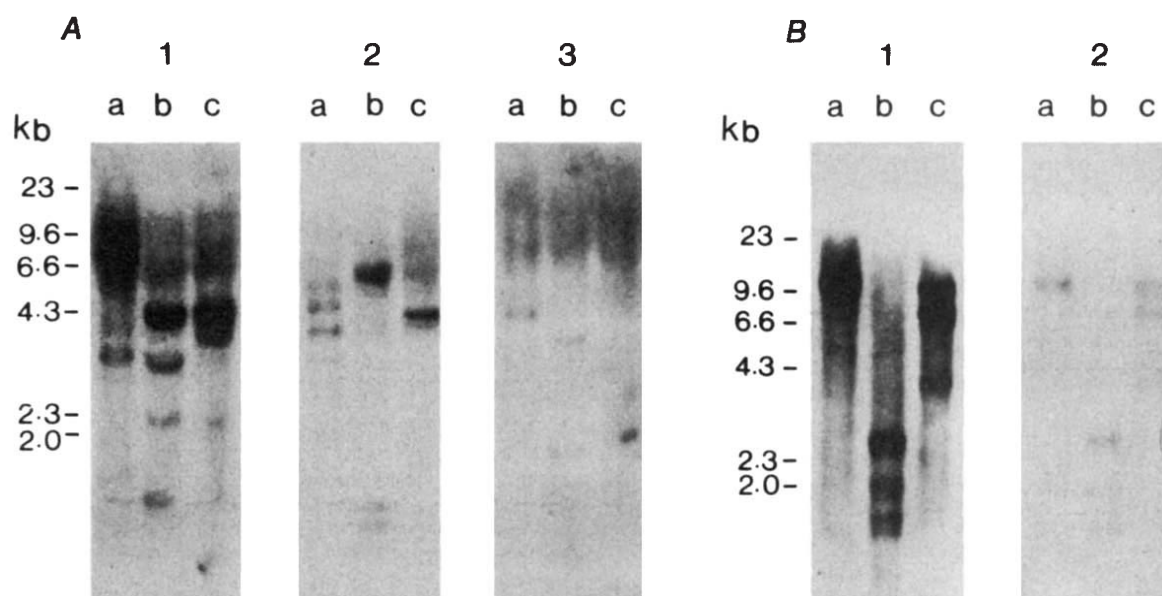


Fig. 4 Restriction map polymorphism in different HIV-2 isolates, and comparison of HIV-2 with SIV. **A**, DNA (20 μ g per lane) from CEM cells infected by the isolate HIV-2_{DUL} (panel 1) or peripheral blood lymphocytes (PBL) infected by the isolates HIV-2_{GOM} (panel 2) and HIV-2_{MIR} (panel 3) was digested with: a, *EcoRI*; b, *PstI*; and c, *HindIII*. Note that much less viral DNA is obtained with HIV-2 isolates propagated on PBL. Hybridization and washing were in stringent conditions, as described in Fig. 2, with 10^6 c.p.m. ml^{-1} of each of the E2 insert (cDNA) and the 5-kb *HindIII* fragment of ROD 4, labelled to 10^9 c.p.m. μg^{-1} . **B**, DNA from HUT 78 (a T lymphoid cell line) cells infected with SIV Mm 142-83; the same amounts of DNA and enzymes were used as for **A**. Hybridization was performed with the same probe as **A**, but in non-stringent conditions, as described (Fig. 1). Washing was for 1 h in $2\times\text{SSC}$, 0.1% SDS at 40°C (panel 1) and after exposure, the same filter was re-washed in $0.1\times\text{SSC}$, 0.1% SDS at 60°C (panel 2). The autoradiographs were obtained after overnight exposure with intensifying screens.

infected patients, whereas the serological cross-reactivity of HIV-1 to 2 is restricted to the core proteins². However SIV and HIV-2 can be distinguished by slight differences in the apparent molecular weight of their proteins². In terms of nucleotide sequence, it also appears that the HIV-2 is very close to SIV. The genomic RNA of SIV (provided by Dr R. Desrosiers) can be detected in stringent conditions (Fig. 2C) by HIV-2 probes corresponding to the LTR and 3' end of the genome (E2) or to the *gag* and *pol* genes (data not shown); in the same conditions, HIV-1 derived probes do not detect the SIV genome (Fig. 2D). In Southern blots of DNA from SIV-infected cells, a restriction pattern clearly different from HIV-2_{ROD} and other isolates is seen (Fig. 4B). All the bands persist after a stringent washing, although the signal is considerably weakened, indicating a sequence homology throughout the genomes of HIV-2 and SIV. It has recently been shown that baboons and macaques could be experimentally infected with HIV-2, providing an interesting animal model for the study of the HIV infection and its preventive therapy (P. Fultz and R. Desrosiers, personal communication). Attempts to infect non-human primates with HIV-1 have only succeeded in chimpanzees, which are not a convenient model.

We have started a survey of the restriction maps of some of the HIV-2 isolates obtained by our group and from this first study it is already apparent that HIV-2, like HIV-1, undergoes restriction-site polymorphism. Figure 4A gives examples of such differences for three isolates, all different one from another and from the cloned HIV-2_{ROD}. It is very likely that these differences at the nucleotide level are accompanied by variations in the amino-acid sequence of the viral proteins, as in the case of HIV-1 (ref. 9).

Recently another group reported the isolation of a human retrovirus from West Africa, apparently highly related to HIV-2, and provisionally named human T-lymphotropic virus type 4 (HTLV-4, ref. 10). This virus displays tropism for CD4⁺ cells, and sera from infected patients detect all the HIV-2 proteins

(F.C., unpublished data). HTLV-4 has not been isolated from AIDS patients, and therefore has been postulated to be non-pathogenic^{10,11}. We feel that this apparent difference in the pathogenicity of HIV-2 and HTLV-4 is due to their means of discovery. That is, HIV-2 was isolated in a survey of AIDS cases in the West Africa, whereas HTLV-4 was found after a large scale serological screening of populations in Senegal for the presence of anti-HIV-1 antibodies and therefore primarily in healthy individual¹¹. As HIV-2 and HTLV-4 infect individuals from neighbouring geographical areas, we think that they will fall into the same group of the HIV family.

Another interest in the characterization of HIV-2 is its possible contribution to the delineation of the domain of the envelope glycoprotein responsible for binding to the surface of the target cells and the subsequent internalization of the virus. This interaction was shown to be mediated by the CD4 molecule itself in the case of HIV-1 (refs 12, 13) and similar studies tend to indicate that HIV-2 uses the same receptor². Thus, although there is wide divergence between the *env* genes of HIV-1 and 2, we may find small homologous domains of the envelopes of the two HIV that could represent candidate receptor-binding site. This site could constitute a target for the attempts of raising a protective immune response against this group of retroviruses.

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A new way of enhancing the thermostability of proteases

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Thermostability of enzyme can be enhanced by single amino acid substitutions^{1,2}. Recent advances in genetic engineering have made it possible to create novel proteins in a predictable manner where structural information for the protein is available. This 'protein engineering'³ has already been used to enhance enzyme thermostability^{4,5}, but it is usually not clear which amino acid substitutions should be made. We consider that the following approach should be helpful in engineering proteins with enhanced thermostability: highly conserved residues should be left unchanged; the sequences of known mesophilic and thermophilic proteins should be used to suggest the kinds of changes likely to increase thermostability⁶; and substitutions should be made that increase internal hydrophobicity and that stabilize helices for strong internal packing. We describe here the use of this approach to alter the thermostability of the thermostable neutral protease of *Bacillus stearothermophilus*, the sequence of which is known^{7,8}. Surprisingly we find

that a single mutation that decreases thermostability can require two mutations that increase stability to compensate for it. The effects on stability are not additive, suggesting cooperativity.

Comparison of primary structures of enzymes of the same function but different origins is useful to determine the essential regions for activity, because active and/or substrate-binding sites are highly conserved in the homologous regions⁹. Amino acid sequences of four neutral proteases from *B. stearothermophilus*⁸, *Bacillus thermoproteolyticus*¹⁰, *Bacillus subtilis*¹¹ and *Bacillus amyloliquefaciens*¹² were therefore aligned and compared (Fig. 1).

The amino acid sequence of thermostable neutral protease from *B. stearothermophilus* was homologous (85%) with that of thermolysin from *B. thermoproteolyticus*. Similarly, the sequences for thermolabile neutral proteases from *B. subtilis* and *B. amyloliquefaciens* were also homologous (89%). In contrast, the homology between the thermostable and thermolabile enzymes was lower (~45%) (Fig. 1). Nine regions were found to be conserved in all four enzymes. These highly homologous regions are likely to be essential for the enzyme activity. In fact, it has been reported that His 234 and Arg 206 are the active and substrate-binding sites of neutral protease (or of thermolysin), respectively¹³. Zn ion is essential for the enzyme activity of thermolysin, and the three protein ligands for Zn (His 145, His 149 and Glu 169)¹³ are conserved in all enzymes (Fig. 1). Glu 146, which promotes the attack of a water molecule on the carbonyl carbon of the substrate¹³, is also conserved in these enzymes (Fig. 1).

Fig. 1 Comparison of amino acid sequences of various extracellular neutral proteases. Amino acid residues are shown by single letters: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. A blank indicates the absence of corresponding amino acid of this position. Enzyme sources are: 1, *Bacillus stearothermophilus*⁸; 2, *Bacillus thermoproteolyticus*¹⁰; 3, *Bacillus subtilis*¹¹; 4, *Bacillus amyloliquefaciens*¹². Homologous sequence regions are surrounded by rectangles. Active and substrate-binding sites of thermolysin are indicated by ○ and □, respectively. Protein ligands for Zn and Ca ions for thermolysin are indicated above the sequence. Substitutions expected to enhance or reduce the thermostability of *B. stearothermophilus* protease in comparison with that of thermolysin from *B. thermoproteolyticus* are indicated above the sequence by + or -, respectively. Vertical arrows, amino acid substitutions in *B. stearothermophilus* protease.



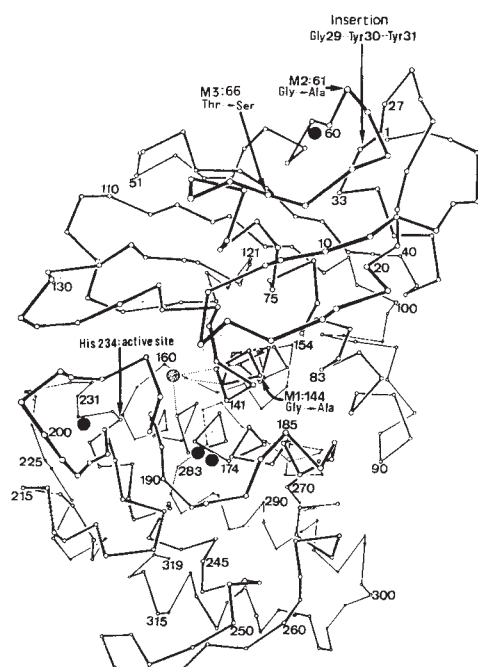


Fig. 2 Three-dimensional structure of thermolysin¹⁴. Open circles, α -carbon positions. Zinc atom is drawn stippled with its three protein ligands shown diagrammatically as broken lines. Four calcium atoms are shown as solid circles. The amino acid number from the NH_2 -terminus is for *B. stearrowthermophilus* neutral protease; originally, Gly-Tyr-Tyr at positions 29-31 are absent in thermolysin.

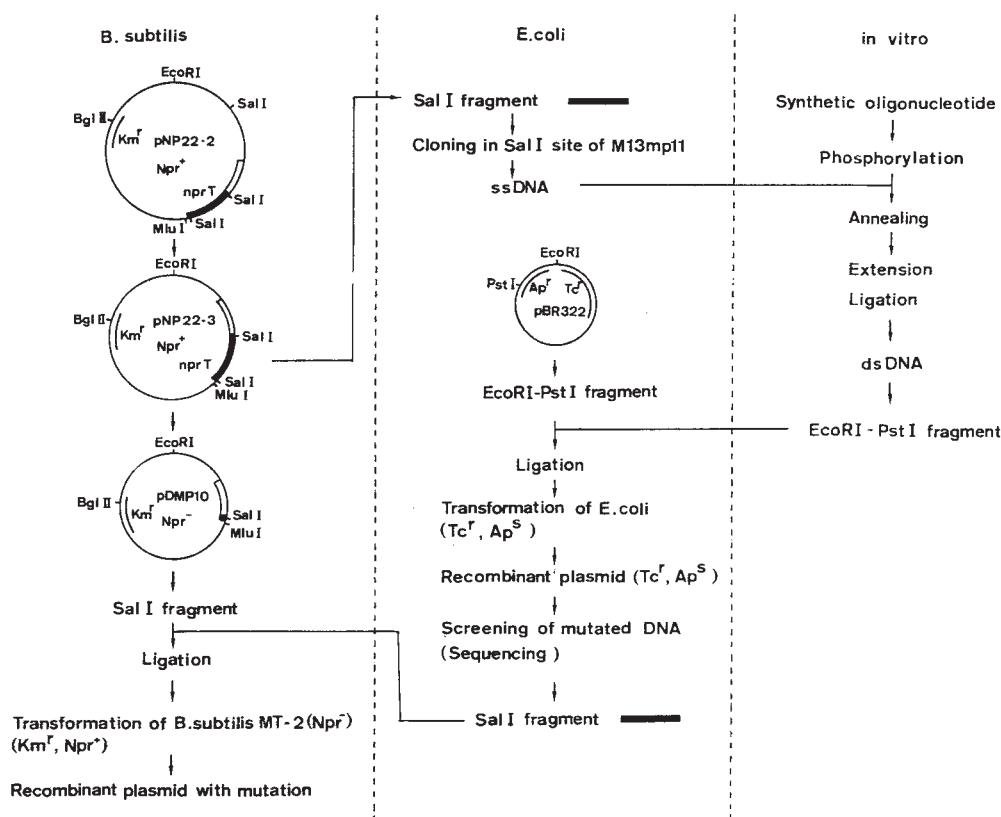


Fig. 3 The scheme of site-directed mutagenesis and DNA construction. The *B. stearrowthermophilus* $nprT^+$ gene has been cloned in pNP22-2, which is a deletion derivative of pNP22-1 (ref. 8). Since the plasmid contains three *SalI* sites, one *SalI* near an *EcoRI* site was eliminated by partial digestion of pNP22-2 with *SalI*, digestion with *Bal31*, and ligation. The plasmid thus obtained is designated pNP22-3. The 888 base pair (bp) *SalI* fragment containing most of the extracellular portion of protease was isolated from pNP22-3 (Npr^+), and the resulting plasmid (Npr^-) was designated as pDMP10. The 888 bp *SalI* fragment was cloned as described¹⁵ in M13mp11, and single-stranded (ss) DNA was isolated. The ss DNA was annealed with a synthetic oligonucleotide whose 5' end had been phosphorylated with polynucleotide kinase. After DNA extension using the primer and ligation, the double-stranded DNA was digested with both *EcoRI* and *PstI*. The *EcoRI*-*PstI* fragment was ligated with large *EcoRI*-*PstI* fragment of pBR322. The ligation mixture was used to transform *Escherichia coli*. Recombinant plasmids were isolated from the transformants (Tc^r , Ap^s). Mutated DNA was screened by either restriction enzyme treatment (mutated primer 5'-CGTCGTGGCGCATGAGT-3', *HhaI* for mutation M1 and 5'-GACCGATGCGGACAC-3', *SfaNI* for mutation M2; asterisks, indicate mismatches and underlined sequences the new restriction site) or colony hybridization¹⁶ and the mutation was confirmed by DNA sequencing. The 888 bp *SalI* fragment with mutation was isolated from the recombinant plasmid, and ligated with *SalI* treated pDMP10. The ligation mixture was used to transform *B. subtilis* MT-2 (Npr^-)⁷ to Km^r Npr^+ . *B. subtilis* MT-2 carrying the mutant plasmid was used to produce extracellular neutral protease.

The comparison of *B. stearothermophilus* protease and thermolysin revealed that the amino acid frames were completely matched except for additional three amino acids (Gly-Tyr-Tyr, 29-31) in the enzyme from *B. stearothermophilus* (Fig. 1). Therefore, the three-dimensional structure of *B. stearothermophilus* protease would be basically similar to the known tertiary structure of thermolysin¹⁴ shown in Fig. 2. Although His 234 (active site), Arg 206 (substrate-binding site), Leu 205, Glu 193 and Asn 162 are far apart in the primary structure of the enzyme (Fig. 1), these amino acid residues are near the active site in the three-dimensional structure (Fig. 2) and are completely conserved in the four neutral proteases tested. Therefore, alteration of amino acids in the highly conserved sequence should be avoided.

We decided the position and species of amino acid to be replaced to give enhanced enzyme thermostability according to the procedure outlined. The amino acid substitutions between *B. stearothermophilus* protease and thermolysin were examined in the light of statistical data on amino acid substitutions which increase thermostability⁶ (Fig. 1). Thermolysin and *B. stearothermophilus* protease contain some amino acids which promote higher thermostability in the first and the second halves, respectively. Therefore, the amino acid substitution in thermostable neutral protease was confined to the first half. The substitution Gly to Ala is available for positions 47, 61 and 144. Since Gly 144 is in the α -helix which combines two domains (Fig. 2), the amino acid substitution Gly 144 to Ala 144 (mutation M1) should increase the internal hydrophobicity, stabilize the α -helix and thus improve the thermostability of the protease. Since it requires only the addition of a methyl group this replacement minimizes interruption of function or internal residue packing arrangements. Another mutation, M3 (Thr 66 to Ser 66) should decrease thermostability of the enzyme. Amino acid substitutions were performed by site-directed mutagenesis (Fig. 3).

Thermostability of wild-type (WT) neutral protease of *B. stearothermophilus* and thermolysin was tested at 75 °C (Fig. 4a). Thermolysin was more thermostable than the WT protease from *B. stearothermophilus*. When M1 mutation (Gly 144 to Ala 144, GGG to GCG) was introduced, the M1 enzyme was found to be more thermostable than WT enzyme (Fig. 4a, b). The M3 mutation (Thr 66 to Ser 66, ACC to TCC) was found to produce fairly thermolabile enzyme (Fig. 4b). The double mutant M13 was constructed, with both the M1 and M3 mutations. Surprisingly, M13 enzyme had the same thermostability as M3. When a different thermostability increasing mutation, M2 (Gly 61 to Ala 61, GGC to GCC), was added to M3, the double mutant enzyme M23 showed the same stability as M3. But when both mutations M1 and M2 were introduced into M3, the stability of the triple mutant enzyme M123 recovered to some extent compared to that of M3 (Fig. 4b). It is interesting that two mutations are required simultaneously to enhance the thermostability of M3 enzyme.

The statistical data in ref. 6 were obtained from many simultaneous replacements of amino acids between thermophilic and mesophilic enzymes. We attempted single amino-acid substitution to test the hypothesis and found that a single substitution does not always enhance thermostability.

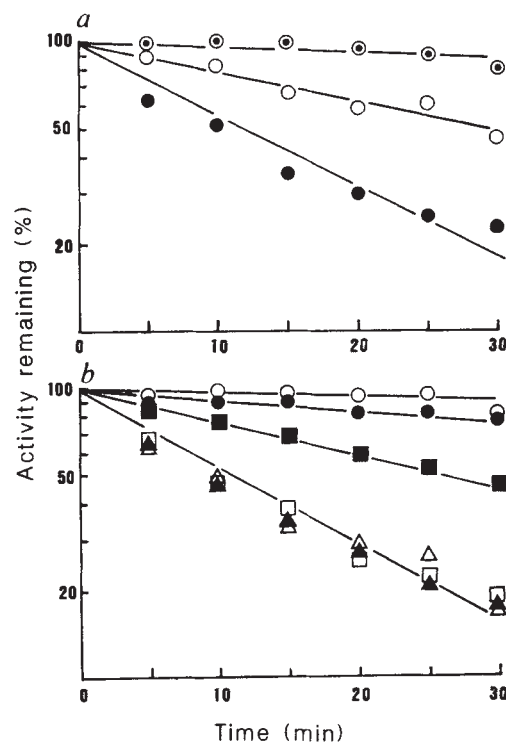


Fig. 4 Thermostability of neutral proteases. Extracellular protease produced by *B. subtilis* carrying *nprT*⁺ gene was purified by the method described previously⁷. Remaining activity after heating at: a, 75 °C; b, 65 °C was assayed as described earlier⁷ and was expressed as the percent of original activity. ○, Thermolysin; ●, wild-type enzyme (WT) from *B. stearothermophilus*; ○, mutant M1; △, mutant M3; ▲, double mutant M13; □, double mutant M23; ■, triple mutant M123.

The thermostability of an enzyme is influenced by many factors, including amino acid sequence, three-dimensional structure, cofactors and pH. The *Bacillus* neutral proteases require Ca ion as the most important enzyme stabilizing factor. Four Ca-binding sites were found in thermolysin (Figs 1 and 2)^{14,17}. Some Ca-binding sites, Asp 141, Asp 188, Glu 193 and Asp 194 are conserved for all proteases examined (Fig. 1). However, a Ca-binding site, Glu 180, of thermostable proteases from *B. stearothermophilus* and *B. thermoproteolyticus* is deleted in the proteases from *B. subtilis* and *B. amyloliquefaciens* (Fig. 1). This might account for the fact that the latter proteases are rather thermolabile. If the amino acid sequence from Thr 177 to Phe 181 were inserted in the *B. subtilis* protease, the enzyme would become more thermostable. Studies on other mutations using this approach are in progress.

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EXAFS study of the zinc-binding sites in the protein transcription factor IIIA

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The protein transcription factor IIIA (TFIIIA) is involved in the synthesis of 5S RNA *in vitro* by RNA polymerase III. It can be isolated from *Xenopus laevis* oocytes as a 7S particle in which the protein is associated with 5S RNA. Recently it has been shown that the native particle contains 7–11 zinc atoms¹. Analysis of the amino-acid sequence of TFIIIA revealed nine similar domains of approximately 30 amino acids, each containing two invariant pairs of histidines and cysteines, which have been implicated as possible binding sites for the zinc atoms. Other regulatory proteins with sequence homology to the zinc-binding domains of TFIIIA have now been reported^{2–4}. Here, we report the results of an EXAFS (extended X-ray absorption fine structure) study of TFIIIA which shows that the coordination sphere of the zinc sites consists of two cysteine and two histidine residues.

EXAFS provides information concerning the number and type of atoms and their bond distances from a metal atom in a metalloprotein^{5,6}. The limitations of the technique include (1) atoms of similar atomic weight (for example oxygen and nitrogen) cannot be distinguished from one another⁷, (2) outer shell distances and number become more unreliable⁸, (3) the EXAFS of an atom that occupies several different sites will be an average of all the sites⁹. If care is taken in the analysis the technique still offers a means of structural characterization of the local environment of the metal sites. The method depends on analysing the oscillations in the absorption or fluorescence spectrum over several hundred electron volts above the metal absorption edge. For biological systems where the metal content is usually low, typically ~1–6 mM, very intense X-ray sources are required and synchrotron radiation is ideal in this respect.

TFIIIA was isolated as a 7S particle using the procedure of Miller *et al.*¹ and the sample was kept in ice before use. The protein concentration was ~2.25 mM in zinc by amino-acid analysis assuming that one zinc atom was present in each repeating unit of the protein. The intensity of the fluorescence signal in the EXAFS measurement is consistent with this value, so it is probable that all nine sites are occupied by zinc atoms.

X-ray absorption spectra were recorded over the energy range corresponding to the zinc K-edge in the fluorescence mode as the fluorescence excitation spectra with individual electronics for each of the four NaI detectors. Data were collected at room temperature at the EXAFS station on the Wiggler beam line at the Daresbury Synchrotron Radiation Source operating at an energy of 2 GeV with an average current of 100 mA. Harmonic contamination was minimized by using a double-crystal Si 220 monochromator¹⁰ so that the EXAFS oscillations would be accurately measured at each position of the monochromator. A total of 28 scans were collected for the TFIIIA and each spectrum

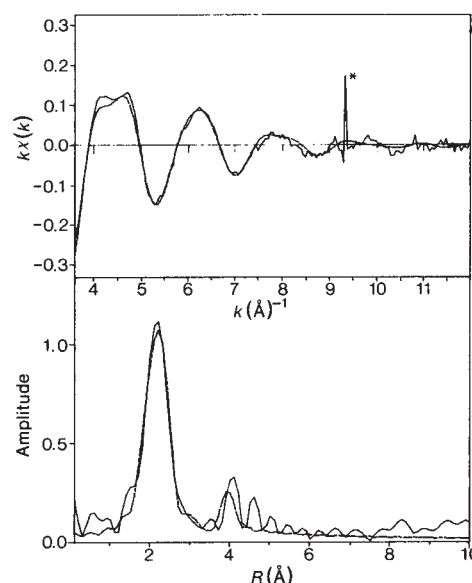


Fig. 1 Zn K-edge EXAFS and Fourier transform of transcription factor IIIA. Continuous lines represent the experimental spectra, broken lines the theoretical simulation. EXAFS is presented as $k\chi(k)$ plotted against k where k is in $\text{\AA}^{-1}$. It should also be noted there is a glitch in the spectrum, indicated by asterisk; this is due to other planes in the silicon crystals of the monochromator diffracting and altering the wavelength content of the beam passing through the sample¹⁹. For the EXAFS measurements the protein solution was loaded into a teflon cell, 5 mm thick and 25×30 mm in cross-section in which a window 4×15 mm had been cut, using a syringe. The sample was contained between mylar windows. $\chi(k)$ is the function describing the oscillations of the EXAFS spectrum, and is multiplied by k to enhance features at high k . k is the momentum of the photoelectron given by: $k = [(E - E_0)2m/\hbar^2]^{1/2}$ where m is its mass, E its energy and E_0 the energy of the absorption edge.

was checked individually, and the output from each detector was inspected before averaging. Approximately half the spectra were rejected because of odd features and jumps occurring in the data which were not reproducible and the remaining spectra were averaged to produce a single spectrum per TFIIIA with a reasonable signal to noise ratio. No radiation damage was observed as no change was seen in the edge of EXAFS between spectra. Any change in the local environment as a result of radiation damage would be expected to manifest itself as a change at least in the XANES (X-ray absorption near edge structure) spectrum. Data analysis using the single-scattering spherical-wave method for calculating the EXAFS with phase shifts derived from *ab initio* calculations^{11–13}, the determination of the quality of fit and the refinement were accomplished as described previously¹¹.

Phase shifts and the calculation procedures were checked against a model compound of known crystal structure, zinc II (1,10-phenanthroline)*bis*(4-toluenethiolate)¹⁴ which was collected in transmission mode at the zinc K-edge (model compound supplied by C. D. Garner, Department of Chemistry, Manchester University). Only the primary co-ordination sphere was used to fit the model. The theoretical EXAFS simulation was produced with two nitrogen atoms at ~2.10 Å and two sulphur atoms ~2.25 Å with Debye-Waller type factors (σ^2) of 0.007 and 0.006 ($\text{\AA}^2$), compared with the crystal structure average of two nitrogen atoms at 2.112(5) Å and two sulphur atoms at 2.261(2) Å. Agreement between the two methods is very good, as the error in fitting the initial shell of atoms for EXAFS is expected to be ± 0.02 Å. Thus the reliability of the EXAFS interpretation for the model gave us confidence in the refinement procedure and in transferring the calculated phase shifts to TFIIIA.

Table 1 Parameters used to simulate the EXAFS associated with the zinc K edge of transcription factor IIIA

	Atom	No	$\sigma^2(\text{\AA}^2)$	$R(\text{\AA})^\dagger$
1st nearest neighbours	N	2	0.0040	2.00
	S	2	0.0045	2.30
2nd and 3rd nearest neighbours	C	4	0.0125	3.07
	C	4	0.0035	3.88

* σ^2 is a Debye-Waller-like factor and is equivalent to $\Delta R_{\text{r.m.s.}}^2$, the mean square deviation of an atom from its mean position.

† The distance of atoms from the zinc atom.

The EXAFS associated with the zinc K-edge of TFI_{II}A, the best theoretical simulation and the corresponding Fourier transforms are shown in Fig. 1, and Table 1 shows the parameters required to produce the simulation. The successful simulation was obtained with zinc co-ordinated by four ligands, two nitrogen atoms at a distance of 2.0 Å and two sulphur atoms at 2.3 Å. It was clear when fitting the data that these two shells on their own did not reproduce the experimental EXAFS and that additional shells were required. Note that the beat region in the EXAFS at $k=4-5 \text{ Å}^{-1}$ (that is, the dip in the peak) and to a lesser extent $k=5.5-7 \text{ Å}^{-1}$ is typical of back-scattering observed for copper and zinc metalloproteins with imidazole co-ordination^{7,13,15}. Thus two shells of carbon atoms were included in the fit and the refined calculation gives distances of 3.07 Å and 3.88 Å. If two carbon and two nitrogen atoms are substituted for the distant four carbons so that the imidazole structure is imitated no improvement in the fit is seen. This is not surprising because the phase shifts for carbon and nitrogen are very similar to each other and it is impossible to distinguish between the two types of atoms at ~4.0 Å. The need to include the two shells of carbon atoms is characteristic of the presence of imidazole groups and leads to confidence in Zn-N (rather than Zn-O) coordination. When the geometry of the imidazole ring is taken into account, the radial distance of the outermost shell is 0.2–0.3 Å shorter than it should be because of the effects of multiple scattering¹⁶, and this is seen in all imidazole and porphyrin structures when the single scattering theory of EXAFS is used^{11,13,15}. Thus the EXAFS suggests that two of the ligands bound to the zinc atom are imidazole groups from histidines in the protein.

The experimental EXAFS is dominated, however, by the phase and the back-scattering amplitude of an atom heavier than nitrogen or oxygen. The overall profile is typical of back scattering from sulphur atoms suggesting that a shell of two sulphur atoms is responsible for most of the EXAFS; when incorporated into the EXAFS simulation of TFI_{II}A it refined to a distance of 2.3 Å. This value agrees well with other known Zn-sulphur coordinated metalloproteins^{15,17,18}. We attempted to simulate the EXAFS with oxygen and/or nitrogen replacing sulphur at its calculated position; the EXAFS oscillations for the lighter atoms were ~180° out of phase with sulphur and did not reproduce the experimental EXAFS of the main peak in the Fourier transform¹⁵.

The simplest simulation of the ligands involved in co-ordination of the zinc atom in TFI_{II}A, that also achieves a good match of the Fourier transforms, consists of two imidazole groups from histidine residues and two sulphur atoms probably from cysteine groups, as originally suggested by Miller *et al.*¹. There is no evidence for the presence of water as an additional ligand. The Debye-Waller-like factors give an indication of thermal and static disorder of the system, and can be used to assess the homogeneity of the zinc sites in TFI_{II}A. The fact that low values are required for the theoretical simulation to match the observed EXAFS suggests that all the zinc atoms are in a similar environment.

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Site-directed mutagenesis reveals role of mobile arginine residue in lactate dehydrogenase catalysis

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The binding of substrates to lactate dehydrogenases induces a marked rearrangement of the protein structure in which a 'loop' of polypeptide (residues 98–110) closes over the active site of the enzyme^{1,2}. In this rearrangement, arginine 109 (a basic residue conserved in all known lactate dehydrogenase sequences and in the homologous malate dehydrogenases³) moves 0.8 nm from a position in the solvent⁴ to one in the active site^{1,5,6} where its guanidinium group resides within hydrogen bonding distance of both the reactive carbonyl of pyruvate and imidazole ring of the catalytic histidine 195 (see Fig. 1). Whilst this feature of the enzyme has been commented upon previously¹, the function of this mobile arginine residue during catalysis has not been tested experimentally. The advent of protein engineering has now enabled us to define the role of this basic residue by substituting it with the neutral glutamine. Transient kinetic and equilibrium studies of the mutant enzyme indicate that arginine 109 enhances the polarization of the pyruvate carbonyl group in the ground state and stabilizes the transition state. The gross active-site structure of the enzyme is not altered by the mutation since an alternative catalytic function of the enzyme (rate of addition of sulphite to NAD⁺), which does not require hydride transfer, is insensitive to the arginine → glutamine substitution.

To make use of site-directed mutagenesis to examine the structure and catalytic mechanism of lactate dehydrogenase (LDH) we have isolated and sequenced the gene from *Bacillus stearothermophilus* and have obtained high levels of expression (about 30% of the soluble protein) of the enzyme in *Escherichia coli*⁷. This prokaryotic enzyme has a primary sequence⁸ and catalytic properties, when activated by fructose-1,6-bisphosphate (FBP)⁹, which are very similar to those of the eukaryotic LDHs from pig and dogfish, the crystal structures of which are known to high resolution^{1,4,5,6}. Furthermore, the amino acid sequences around the site of catalysis and the coenzyme-binding domain are almost identical⁸. These factors give us confidence that the three-dimensional structure of the functional regions of the *B. stearothermophilus* enzyme are the same as those of the eukaryotic enzymes—indeed, the preliminary crystallographic structure of LDH from *Lactobacillus casei* shows that this prokaryotic, FBP-stimulated enzyme is very similar to eukaryotic LDH (M. Buehner, personal communication).

To examine the function of arginine 109 in catalysis, this residue was replaced by glutamine in order to retain bulk and hydrophilicity, but to remove the positive charge. To do this, a

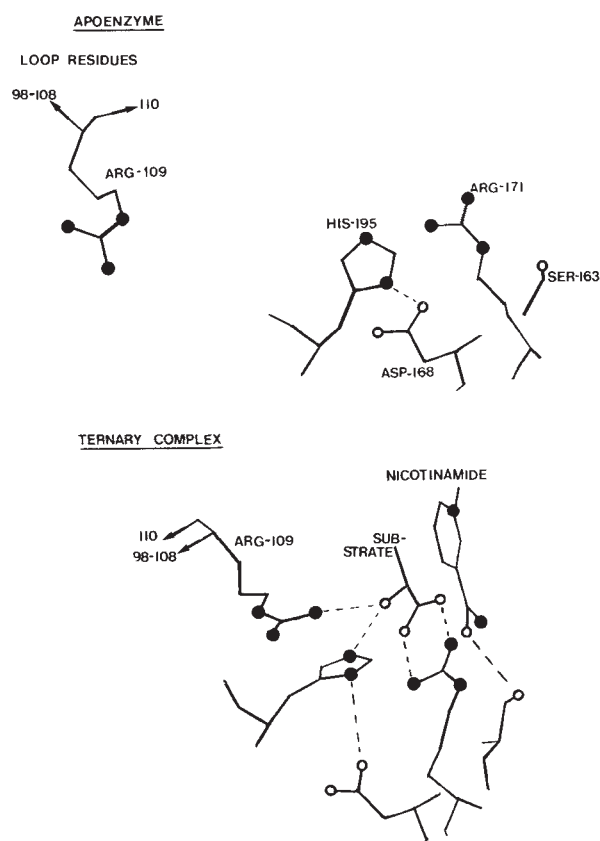


Fig. 1 The movement of arginine 109 on the binding of substrates to LDH. Two views of LDH (generated on FRODO graphics) from identical angles, centred on the active-site histidine 195. The structures are the gene-derived *B. stearothermophilus* sequence⁷ built into (JJH unpublished) the apoenzyme structure of dogfish M₄ enzyme⁴ and the ternary complex of the pig H₄ S-lac-NAD⁺ enzyme¹ (using coordinates from the Brookhaven Protein Data Bank files). The scaled projections emphasize the 0.8 nm movement of arginine 109 into the active-site on closure of a mobile polypeptide loop (residues 98–100) against the surface of the protein.

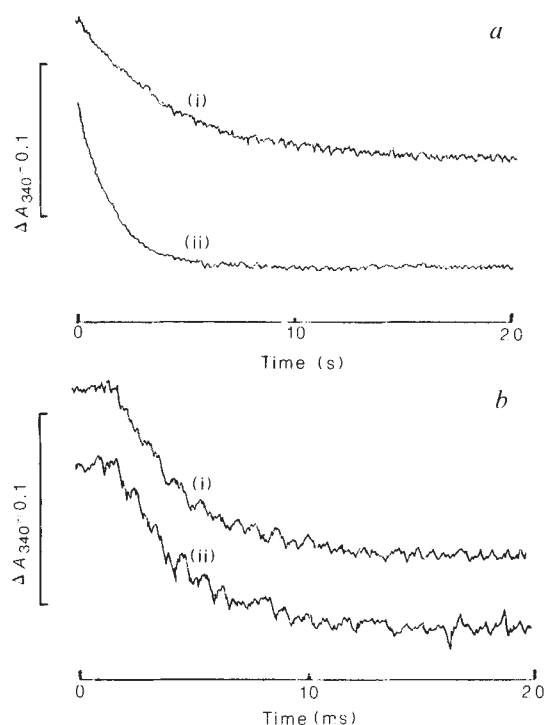


Fig. 2 The coenzyme deuterium isotope effect on the kinetics of wild-type (b) and mutant (a) LDH. A solution containing 40 μ M enzyme subunits, 40 μ M NADH or NAD²H (nicotinamide-4-²H) and 5 mM FBP was rapidly mixed with an equal volume of a solution containing 20 mM pyruvate and 5 mM FBP in a stopped flow spectrophotometer (Hi-Tech, Salisbury). The solutions were buffered with 20 mM triethanolamine HCl pH6 and the experiment was performed at 25 °C. The traces show the reduction in absorbance at 340 nm as the binary complex, enzyme-NADH (or enzyme-NAD²H), is converted to enzyme-NAD⁺ in a single-turnover reaction by the addition of pyruvate. The results for the mutant enzyme with NAD²H and NADH are shown in a(i) and a(ii), respectively, and those for the wild-type in b(i) and b(ii). In each case the experiment produced a single exponential decay with rates of 0.18 s⁻¹ a(i), 0.55 s⁻¹ a(ii) and 245 s⁻¹ b(i) and b(ii).

22-base oligonucleotide was synthesized with a single mismatch at the second base of the arginine-109 codon (GGC → GTC). This oligonucleotide was used as a primer to generate the mutated LDH gene in an M13 mp8 vector, as described by Winter *et al.*¹⁰. This gene was then sequenced (to ensure that only the directed mutation had arisen), excised and inserted into a high-expression TAC vector¹¹. This construct was used to transform *E. coli* TG2 cells (a *recA*⁻ form of TG1¹²).

In the transformed cells, grown overnight in a 2 × YT medium⁷, 30% of the soluble protein (as judged by polyacrylamide gel electrophoresis) was the mutant LDH; this was purified by affinity chromatography on oxamate-Sepharose as described previously¹³. Table 1 shows some of the catalytic and ligand-binding properties of the purified mutant enzyme in comparison with those of the native. Substitution of the loop arginine leads to a 30-fold weakening of pyruvate *K_m* and a 420-fold reduction (from 250 s⁻¹ to 0.6 s⁻¹) of *k_{cat}*. There is little change in the thermodynamics of coenzyme-binding (measured directly from NADH anisotropy⁹), the *pK* of histidine 195 which controls substrate binding or the transfer of information between the regulatory (FBP-binding) site and the substrate-binding site; occupation of the former still leads to an increase in affinity of the latter (50-fold in the native and 30-fold in the mutant enzyme).

The advantage of mutating LDH (apart from the remarkable

yield of isolatable mutant enzyme¹⁴) is that many techniques are developed to distinguish the individual steps in catalysis—including coenzyme-binding, protonation, substrate-binding, protein conformational change and hydride ion transfer^{15,16}—so that mutations can be interpreted in terms of their effects on individual reaction steps, rather than the often complex steady-state parameters of *k_{cat}* and *K_m*. The severity of the effect on turnover rate led us to question whether the removal of the arginine 109 residue had changed the nature of the process limiting the maximal velocity of the enzyme. In the native enzyme the slowest step is a structural rearrangement of the enzyme-NADH-pyruvate complex just prior to substrate reduction. This was shown (as for the eukaryote enzymes²⁰) by the catalytic rate being the same for the transfer of either a deuteride or hydride ion from C-4 of the nicotinamide ring of the coenzyme to C-2 of pyruvate at the active-site and the absence of a 'burst' in production of NAD⁺.

When this experiment was performed on the mutant enzyme with NAD²H, the reaction rate was three times slower than with NADH itself (see Fig. 2). Primary isotope effects of 3–6-fold are typical for dehydrogenases^{21,22} and we conclude that in the mutant enzyme hydride-ion transfer occurs at 0.6 s⁻¹ at 25 °C and is rate-limiting. In the native enzyme the rate limiting step (250 s⁻¹) is a rearrangement of protein structure with *k_H*/*k_D* = 1.0 ± 0.1. In a kinetically complex reaction a process with an

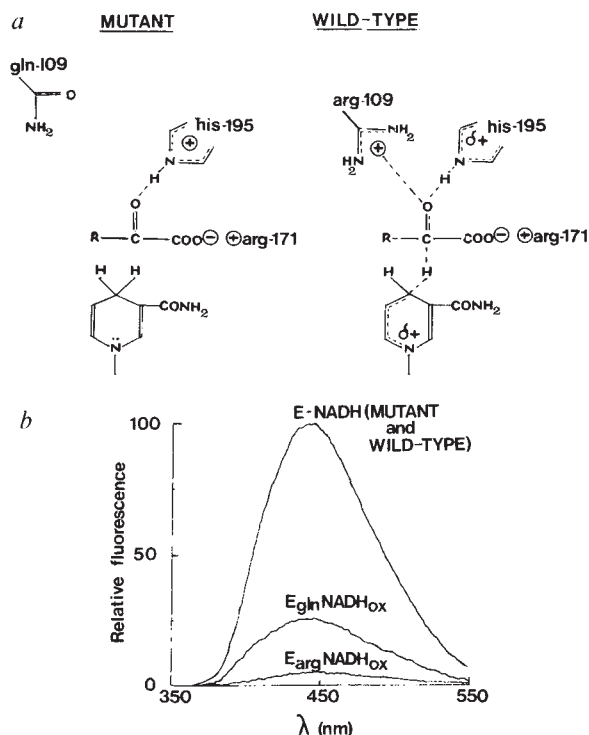


Fig. 3 Changes in coenzyme fluorescence on binding oxamate to the enzyme-NADH complex *a*, Proposed structures in the active site of LDH when both NADH and oxamate are bound to the mutant and wild-type enzymes. The glutamine amide is further from the substrate carbonyl than the guanidinium group. The diagrams emphasize the shift in the electronic structure of the dihydronicotinamide ring in the wild-type enzyme, due to the greater polarization of the substrate carbonyl by arginine 109. Coenzyme fluorescence-emission spectra which reflect this effect²⁴ are shown in *b*. The spectra were taken at an excitation wavelength of 340 nm and the emission was scanned between 350 nm and 550 nm. The concentrations of enzyme (subunits), NADH and FBP were 15 μ M, 10 μ M and 5 mM, respectively and the buffer contained 20 mM triethanolamine-HCl at pH 6. The fluorescence enhancement (35%) and blue shift (450 nm to 440 nm at the peak) when NADH bound to the enzyme was the same for the mutant and wild-type binary complexes. However, addition of a saturating concentration of oxamate (47 mM) to the mutant enzyme-NADH complex gave a quench of 75% whereas the wild-type complex (in 8 mM oxamate) showed a 95% quench (the coenzyme is five times more fluorescent in the mutant ternary complex).

intrinsic $k_H/k_D = 3$ and a rate of 750 s^{-1} would still give $k_{ti}/k_D = 1$, with a preceding protein-isomerization rate of 250 s^{-1} . Thus hydride transfer in the mutant is at least $750/0.6 = 1,200$ times slower than in the wild-type enzyme. Therefore compared to glutamine, arginine 109 reduces the size of the activation energy barrier for hydride transfer by more than 4.2 kcal.

It has been suggested on the basis of crystal structures¹ that arginine 109 destabilizes the enzyme-NADH-pyruvate complex when the loop is closed over the active site, owing to charge repulsion between arginine 109 and the protonated histidine 195 (Fig. 1). This predicts that the binding of oxamate in the ternary complex (in which the histidine is protonated and the 98-110 loop is closed^{2,16,23}) should be tighter in the mutant enzyme, owing to the absence of charge repulsion from arginine-109. This suggestion is not tenable, since Table 1 shows that the binding of oxamate and pyruvate is weaker: the arginine residue stabilizes the NADH-containing ternary complex by 1.6-1.9 kcal. This experimental result is consistent with the observation that oxamate (isoelectronic and isosteric analogue of pyruvate) triggers loop-closure in the native enzyme¹⁶ and thus NADH and oxamate bind more tightly when the loop is down

Table 1 Comparison of the properties of native and mutant (Arg-109 $\rightarrow$ Gln) enzymes

	Native LDH	Mutant LDH
(1) K_d NADH	1.5 μ M	1.5 μ M
(2) K_m pyruvate (unactivated)	2 mM	30 mM
K_m pyruvate (activated)	0.04 mM	1.0 mM
Activation factor (pyruvate)	50	30
(3) k_{cat}	250 s^{-1}	0.6 s^{-1}
(4) K_d oxamate (unactivated)	1.5 mM	25 mM
K_d oxamate (activated)	0.03 mM	1 mM
Activation factor (oxamate)	50	25
(5) pK of histidine 195 in E-NADH	6.4	6.5
(6) Addition of SO_3^- to E-NAD ⁺		
K_d	4 μ M	6 μ M
k_{on}	$0.94 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	$1.22 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$
(7) Thermal stability ($t_{1/2}$ 90 °C)	7.0 min	5.0 min

Unless stated, all parameters were measured at 25 °C in 20 mM triethanolamine-HCl (pH 6). (1) Measured from NADH fluorescence anisotropy⁹. (2) Determined in assay solutions containing saturating (0.2 mM) NADH. Activation factor is the ratio of K_m determined in the absence and presence of 5 mM FBP (the allosteric activator)⁹. (3) The maximum single-turnover rate at saturating NADH and pyruvate (experimental conditions of Fig. 2). (4) Determined fluorimetrically⁹ in presence and absence of 5 mM FBP. (5) Determined for the bacterial enzyme-NADH complexes (6 μ M sites, 5 μ M NADH) in 50 mM TEA pH 6-8¹⁷. (6) K_d (apparent) at pH 7.2, 15 μ M enzyme sites, 0.5 mM NAD⁺, 5 mM FBP. k_{on} is from the variation in first-order rate constant with sulphite (75 μ M to 300 μ M) in stopped-flow experiments under the same conditions¹⁸. The non-enzymatic rate¹⁹ is $2,400 \text{ M}^{-1} \text{ s}^{-1}$. (7) Temperature stability was measured by incubating the 2 μ M enzyme subunits at 90 °C in the presence of 5 mM FBP and assaying the residual enzyme activity.

and the arginine residue is in the active site.

If the role of arginine 109 is not to destabilize the enzyme-NADH-pyruvate complex, then an alternative means by which it stimulates the rate of hydride ion-transfer must be sought. One possibility is that the residue is able to enhance the polarization of the carbonyl group of pyruvate over that achieved by histidine 195 alone (Fig. 1). In their pioneering work on LDH, Winer and Schwert²⁴ suggested that pyruvate and oxamate are able to quench the fluorescence of enzyme-bound NADH by causing a partial transfer of the hydride ion from the nicotinamide C-4 to the substrate C-2 (Fig. 3a) to generate in the ground state a coenzyme structure which more resembles the unfluorescent NAD⁺. The extent of this partial transfer of charge, and resultant fluorescence quench, will depend on the degree of polarization of the substrate carbonyl. Figure 3b shows the NADH fluorescence spectra in the enzyme-NADH and enzyme-NADH-oxamate complexes of the wild-type and mutant LDHs. The result demonstrates that the fluorescence-quench on adding oxamate is far less pronounced in the mutant and provides experimental evidence that arginine 109 increases the polarity of the carbonyl bond of the substrate in the ground state (a function speculated upon by Grau *et al.* in a proposed mechanism for pyruvate reduction¹).

An assumption of the 'mutagenesis' approach is that, in making the substitution, there are no widespread and uninterpretable effects on other regions of the protein²⁵. One control is to determine the three-dimensional structures of the mutant enzyme, but a rapid and more sensitive control is to measure a second catalytic function of the enzyme which is expected to be independent of the mutation. LDH catalyses the addition of sulphite (SO_3^{2-}) to NAD⁺ in the active site of the enzyme. This reaction tests several characteristics of the active site but does not require hydride-ion transfer. It depends on histidine 195 being protonated and NAD⁺ being activated and orientated to facilitate covalent attachment of the sulphite ion to C-4 of the nicotinamide ring of NAD⁺ through a nucleophilic attack^{2,18}. Table 1 shows the mutant enzyme catalyses NAD-sulphite formation slightly faster than the wild-type in contrast to the $>1,200$ -fold reduction in the rate of hydride transfer. Additional evidence that the protein structure is not significantly perturbed by the mutation is its lack of effect upon the temperature stability of the enzyme (Table 1).

In conclusion, the substitution of glutamine for arginine-109 (conserved in all 16 known LDHs and all 4 known MDHs) has changed the properties of the enzyme in a limited but mechanistically interpretable way. We suggest that the binding of the anionic pyruvate at the active site neutralizes some of the positive charge in this region, triggering loop closure and exploiting the favourable hydrophobic contacts^{2,16} made between the loop and the body of the protein. This brings arginine 109 close to the substrate carbonyl (an example of a substrate-induced conformational change). Our data show that the relative enzyme-substrate ground state stabilization of the arginine 109 containing complex, over that containing glutamine, is 1.6–1.9 kcal, while the relative transition state stabilization is 4.2 kcal. In the ground state the polarization of the carbonyl bond of the substrate appears to be enhanced by arginine 109 (Fig. 3*b*). The greater effect of the loss of arginine 109 on the transition state stability is consistent with the carbonyl bond becoming more polarized as a negative charge develops on the carbonyl oxygen when C-2 goes from sp^2 to sp^3 hybridization.

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Isolation of cDNA clones encoding the 20K non-glycosylated polypeptide chain of the human T-cell receptor/T3 complex

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Nature **321**, 431-434 (1986).

THREE sequencing errors have been found in the human T3- ϵ nucleotide sequence published in Fig. 3b. These errors have been corrected in the sequence shown below at the positions indicated by vertical arrows. The putative transmembrane region is underlined. The predicted amino acid sequence ends after residue 185 instead of the reported residue 211.

[illegible]

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Protein kinase C activation induces conductance changes in *Hermissenda* photoreceptors like those seen in associative learning

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Nature 319, 220-223 (1986).

PANEL *c* in Fig. 3 of this letter was incorrectly drawn. With the ordinate scale corrected the figure reads:

Corrigenda

A complimentary DNA clone for a macrophage-lymphocyte Fc receptor

Victoria A. Lewis, Terry Koch, Helen Plutner & Ira Mellman
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A LATE correction to Fig. 1b in this letter was omitted. The nucleotide numbering for pFCrAb should read 805–1421, not 805–1385 as shown. The figure appears correctly on reprints. The insert is of 6/6 base pairs, not 580 as in paragraph 2 of the letter.

